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(12) **Patent Application:** (11) **CA 2108113**

(54) DNA SEQUENCE ENCODING ENZYMES OF CLAVULANIC ACID BIOSYNTHESIS

(54) SEQUENCE D'ADN CODANT POUR DES ENZYMES DE LA SYNTHESE DE L'ACIDE CLAVULANIQUE

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ABSTRACT:
24
ABSTRACT
DNA sequences are provided which encode the enzymes required for clavulanic acid synthesis. A process is provided for producing clavulanic acid in a transformant of a non-clavulanate-producing host.

Canadian Patents

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Patent Document Number 2108113 :
DNA SEQUENCE ENCODING ENZYMES OF CLAVULANIC ACID BIOSYNTHESIS

SEQUENCE D'ADN CODANT POUR DES ENZYMES DE LA SYNTHESE DE L'ACIDE CLAVULANIQUE

CLAIMS:

19
The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows:

1. An isolated genomic DNA molecule comprising the nucleotide sequence of Figure 2 (Sequence ID No.:1).

2. An isolated DNA molecule having the nucleotide sequence of nucleotides 2033 to 13636 of Figure 2 (Sequence ID No.:20).

3. An isolated DNA molecule having the nucleotide sequence of nucleotides 109 to 1764 of Figure 2 (Sequence ID No.:21).

4. An isolated DNA molecule having the nucleotide sequence of nucleotides 2216 to 3937 of Figure 2 (Sequence ID No.:22).

5. An isolated DNA molecule having the nucleotide sequence of nucleotides 3940 to 5481 of Figure 2 (Sequence ID No.:23).

6. An isolated DNA molecule having the nucleotide sequence of nucleotides 5654 to 6595 of Figure 2 (Sequence ID No.:24).

7. An isolated DNA molecule having the nucleotide sequence of

(Sequence ID No.:25).

8. An isolated DNA molecule having the nucleotide sequence of nucleotides 7895 to 9076 of Figure 2
(Sequence ID No.:26).

9. An isolated DNA molecule having the nucleotide sequence of nucleotides 9241 to 10908 of Figure 2
(Sequence ID No.:27).

10. An isolated DNA molecule having the nucleotide sequence of nucleotides 10998 to 12296 of Figure 2
(Sequence ID No.:28).

11. An isolated DNA molecule having the nucleotide sequence of nucleotides 12622 to 13365 of Figure 2
(Sequence ID No.:29).

12. An isolated DNA molecule having the nucleotide sequence of nucleotides 13769 to 14995 of Figure 2
(Sequence ID No.:30).

13. An isolated DNA molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 10.

14. An isolated DNA molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 11.

15. An isolated DNA molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 12.

16. An isolated DNA molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 13.

17. An isolated DNA molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 14.

21

18. An isolated DNA molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 15.

19. An isolated DNA molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 16.

20. An isolated DNA molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 17.

21. An isolated DNA molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 18.

22. An isolated DNA molecule comprising a nucleotide sequence encoding the amino acid sequence of Figure 19.

23. An isolated protein having the amino acid sequence of Figure 10.

24. An isolated protein having the amino acid sequence of Figure 11.

25. An isolated protein having the amino acid sequence of Figure 12.

26. An isolated protein having the amino acid sequence of Figure 13.

27. An isolated protein having the amino acid sequence of Figure 14.

28. An isolated protein having the amino acid sequence of Figure 15.

22

29. An isolated protein having the amino acid sequence of Figure 16.

30. An isolated protein having the amino acid sequence of Figure 17.

31. An isolated protein having the amino acid sequence of Figure 18.

32. An isolated protein having the amino acid sequence of Figure 19.

33. A recombinant vector comprising a DNA molecule in accordance with any of claims 1 to 22.

34. A host transformed with a recombinant vector comprising a DNA molecule in accordance with any of claims 1 to 22.

35. A host transformed with a recombinant vector in accordance with claim 2 wherein the host is a Streptomycete.

36. A host in accordance with claim 35 which is S. lividans.

37. A process for producing clavulanic acid in a non-clavulanate-producing host comprising transforming the host with a DNA molecule in accordance with claim 2 and culturing the host under suitable conditions to produce clavulanic acid.

38. A process for producing clavulanic acid in accordance with claim 37 wherein the host is S. lividans.

39. A process for enhancing clavulanic acid production in a clavulanate-producing host comprising

23 transforming the host with a DNA molecule comprising a nucleotide sequence encoding one or more of the enzymes of the clavulanate synthetic pathway.

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DNA SEQUENCE ENCODING ENZYMES OF CLAVULANIC ACID BIOSYNTHESIS

This invention relates to methods for the production of the antibiotic, clavulanic acid.

Background of the invention

Clavulanic acid is a broad spectrum beta-lactamase inhibitor and is an important antibiotic for the treatment of infectious diseases. It is produced commercially by the gram-positive mycelial prokaryote Streptomyces clavuligerus, which also produces the β-lactam antibiotics penicillin N, deacetoxy cephalosphorin C and cephamycin C. Until recently, however, the pathway employed for clavulanic acid biosynthesis was much less well understood than the pathways leading to these other antibiotics.

Without knowledge of the pathway for clavulanic acid biosynthesis, it was not possible to isolate the genes coding for the key enzymes and to manipulate these genes to increase antibiotic yield or permit production of the antibiotic in heterologous systems.

One of the earliest enzymes of the pathway to be purified and characterised was clavaminic acid synthase. Two isozymes have now been identified and characterised (Marsh et al., (1992), Biochem., Vol. 31, pp. 12648-657).

European Patent Application 0349121 describes a DNA restriction fragment encoding a portion of the genetic information involved in clavulanic acid synthesis but provides no sequence information.

Until the work of the present inventors, the complete complement of genes required for clavulanic acid synthesis had not been identified. The present inventors have now isolated, cloned and sequenced an 11.6 kb genomic DNA sequence from S. clavuligerus which codes for eight proteins and enables the production of clavulanic

acid by transformants of non-clavulanic-producing organisms.

Summary of the Invention

An isolated genomic DNA molecule is provided comprising the nucleotide sequence set out in Figure 2. A process is provided for producing clavulanic acid in a transformant of a non-clavulanate-producing host.

Description of Drawings

The invention, as exemplified by a preferred embodiment, is described with reference to the accompanying drawings in which:

Figure 1 shows the N terminal amino acid sequence of CLA and the nucleotide sequence of a probe (Sequence ID No.:2) directed to the underlined region of the sequence.

Figure 2 (2-1 to 2-10) shows the nucleotide sequence (Sequence ID No.:1) of a 15 kb genomic DNA fragment from S. clavuligerus. The sequences of the ten ORFs within the fragment are shown in upper case letters and the intergenic regions are shown in lower case letters. The locations of the beginning and end of each ORF are also indicated directly above the nucleotide sequence. Asterisks above the sequence indicate the EcoR1 sites which mark the beginning and end of the portion of the DNA sequence which contains all the genetic information for clavulanic acid synthesis.

Figure 3 shows the location of the open reading frames downstream from pcbC.

Figure 4 shows a partial restriction map of the DNA sequence of Figure 2 in the region surrounding cla (ORF4).

Figure 5 shows a shuttle vector used for disruption of the ola gene.

Figure 6 shows a photograph of an agar plate bearing cultures of S. lividans transformants.

Figure 7 shows an alignment of the amino acid sequence of CLA (S. clavuligerus CLA) with those of E. Coli agmatine ureohydrolase (E. Coli AUH), yeast arginase (yeast ARG), rat arginase (rat ARG) and human arginase (human ARG).

Figure 8 shows a Southern blot of NcoI digests of genomic DNA from five presumptive mutants (lanes 1-5) and from wild-type S. clavuligerus (lane 6). Panel A : membranes probed with cla-specific probe. Panel B : membranes probed with ter-specific probe.

Figure 9 shows restriction enzyme maps of S. clavuligerus DNA inserts in cosmids. A. Restriction enzyme map of cosmid K6L2. B. Partial restriction enzyme map of cosmid K8L2. C. Restriction map of cosmids K6L2 and K8L2 indicating location of pcbC gene in relation to cla. D. The 2.0 kb NcoI fragment encompassing the cla gene used in generating nested deletions for sequencing. Abbreviations: Ba, BamHI; B,BglII; E,EcoRI; K,KpnI; N, NcoI; S,SalI; and Sm,SmaI.

Figure 10 shows the deduced amino acid sequence (Sequence ID No.:3) of ORF1 of Figure 2.

Figure 11 shows the deduced amino acid sequence (Sequence ID No.:4) of ORF2 of Figure 2.

Figure 12 shows the deduced amino acid sequence (Sequence ID No.:5) of ORF3 of Figure 2.

Figure 13 shows the deduced amino acid sequence (Sequence ID No.:6) of ORF4 of Figure 2.

Figure 14 shows the deduced amino acid sequence (Sequence ID No.:7) of ORF5 of Figure 2.

Figure 15 shows the deduced amino acid sequence (Sequence ID No.:8) of ORF6 of Figure 2.

Figure 16 shows the deduced amino acid sequence (Sequence ID No.:9) of ORF7 of Figure 2.

Figure 17 shows the deduced amino acid sequence (Sequence ID No.:10) of ORF8 of Figure 2.

Figure 18 shows the deduced amino acid sequence (Sequence ID No.:11) of ORF9 of Figure 2.

Figure 19 shows the deduced amino acid sequence (Sequence ID No.:12) of ORF10 of Figure 2.

Detailed description of the Invention

Production of penicillin and cephamycin antibiotics in S. clavuligerus starts with the conversion of lysine to α-aminoadipic acid (Madduri et al., (1989), J. Bacteriol., v. 171, pp. 299-302; (1991), J. Bacteriol., v. 173, pp. 985-988). α-Aminoadipic acid then condenses with cysteine and valine to give δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) by the action of aminoadipyl-cysteinyl-valine synthetase (ACVS). ACV is converted by isopenicillin N synthase (IPNS) to isopenicillin N, and, through a series of reactions, to deacetoxycephalosporin C and ultimately to cephamycin C (Jensen et al., (1984), Appl. Microbiol. Biotechnol., v. 20, pp 155-160).

The ACVS of S. clavuligerus has been purified and partially characterized by three separate groups, and estimates of its molecular weight vary from 350,000 to 500,000 Da (Jensen et al., (1990) J. Bacteriol., v. 172, pp. 7269-7271; Schwecke et al., (1992); Eur. J. Biochem., v. 205, pp. 687-694; Zhang and Demain, (1990), Biotech Lett., v. 12, pp. 649-654). During their purification, Jensen et al. observed a 32,000 Da protein which co-purified with ACVS despite procedures which should remove small molecular weight components. It has now been found that this protein is not related to ACVS but rather to clavulanic acid biosynthesis. It has been designated CLA.

In accordance with one embodiment of the invention, the present inventors have identified, cloned and sequenced the gene (cla) encoding this protein.

In accordance with a further embodiment of the invention, the inventors have cloned and sequenced a 15 kb stretch of genomic DNA from S. clavuligerus which includes the cla gene. Within this 15 kb sequence, the inventors have identified an 11.6 kb DNA fragment which,

when introduced into the non-clavulanate producer S. lividans as described in Example 4, enabled that species to produce clavulanic acid. This indicates that the 11.6 kb fragment contains all the genetic information required for clavulanate production.

As will be understood by those skilled in the art, the identification of the DNA sequence encoding the enzymes required for clavulanate synthesis will permit genetic manipulations to modify or enhance clavulanate production. For example, clavulanate production by S. clavuligerus may be modified by introduction of extra copies of the gene or genes for rate limiting enzymes or by alteration of the regulatory components controlling expression of the genes for the clavulanate pathway.

Heterologous organisms which do not normally produce clavulanate may also be enabled to produce clavulanate by introduction, for example, of the 11.6 kb DNA sequence of the invention by techniques which are well known in the art, as exemplified herein by the production of S. lividans strains capable of clavulanate synthesis. Such heterologous production of clavulanic acid provides a means of producing clavulanic acid free of other contaminating clavams which are produced by S. clavuligerus.

Suitable vectors and hosts will be known to those skilled in the art; suitable vectors include pIJ702, pJ08929 and pIJ922 and suitable hosts include S. lividans, S. parvulus, S. griseofulvus, S. antibioticus and S. lipmanii.

Additionally, the DNA sequences of the invention enable the production of one or more of the enzymes of the clavulanate pathway by expression of the relevant gene or genes in a heterologous expression system.

The DNA sequences coding for one or more of the pathway enzymes may be introduced into suitable vectors and hosts by conventional techniques known to those skilled in the art. Suitable vectors include pUC118/119

and pET-11 and suitable hosts include many organisms, including E. coli strains such as MV1190 and BL21(DE3).

An oligonucleotide probe based on the N-terminal amino acid sequence of CLA was constructed as shown in Figure 1 and was used to isolate the gene coding for the protein from S. clavuligerus, as described in Example 1.

The gene was found to be located in the S. clavuligerus chromosome about 5.7 Kb downstream of pcbC, the gene which encodes isopenicillin N synthase. The gene contains a 933 bp open reading frame (ORF), encoding a protein of molecular weight 33,368. The deduced amino acid sequence was compared to database sequences and showed greatest similarity to enzymes associated with arginine metabolism, notably agmatine, ureohydrolase and arginases.

When an internal fragment of the cla gene was labelled and used to probe restriction endonuclease digests of genomic DNA from a variety of other Streptomyces and related species, evidence of homologous sequences was seen only in other clavulanic acid or clavam metabolite producers, including Streptomyces jumonjinensis, Streptomyces lipmanii (7) and Streptomyces antibioticus. No cross reactivity was seen to the β-lactam producing species Nocardia lactamdurans, Streptomyces griseus or Streptomyces cattleya, nor to any of a variety of other Streptomyces species which do not produce β-lactam compounds, including S. fradiae ATCC 19609, S. venezuelae 13s and S. griseofulvus NRRL B-5429.

Disruption of the cla gene, as described in Example 3, led to loss of the ability to synthesise clavulanic acid.

A 15 Kb DNA sequence extending downstream from pcbC was cloned and sequenced as described in Example 5. The nucleotide sequence is shown in Figure 2. When this sequence information was analysed for percent G + C as a function of codon position (Gibb et al., (1984), Gene, v. 30, pp. 157-166), ten complete ORFs were evident, as

FIGURE 2 - 6

```
8161 CGAGGTGCGC GAGGCCGTCG CCCGGGCCCT CGGGCTGCCG GAGGCGGAGA TGCTGATCGC 8220
8221 CTCCACCGGG GTGATCGGCC GGCAGTACCC GATGGAGACC ATCCGGGAGC ACCTCAAGAC 8280
8281 GCTGGAGTGG CCCGCCCGGG ACGGCGGCTT CGACCGCGCG GCCCGCGCCA TCATGACGAC 8340
8341 CGACACCCGC CCCAAGGAGG TCCGGGTCAG CGTCGGCGGG GCGACCCTCG TGGGCATCGC 8400
8401 CAAGGGCGTC GGCATGCTGG ACCCCGACAT GGCGACGCTG CTGACCTTCT TCGCCACGGA 8460
8461 CGCCCGGCTG GACCCGGCCG AGCAGGACCG CCTCTTCCGC CGGGTCATGG ACCGCACCTT 8520
8521 CAACGCGGTC AGCATCGACA CCGACACCTC CACCAGCGAC ACGGCCGTGC TGTTCGCCAA 8580
8581 CCGCCTGGCG GGCGAGGTCG ACGCCGGGGA GTTCGAGGAG GCGCTGCACA CGGCGCCGCT 8640
8641 GGCCCTGGTC AAGGACATCG CGAGCCACGG CGAGGGCGCG GCCAAGCTGA TCGAGGTCCA 8700
8701 GGTCACCGGC CCCCGCGACG ACGCCCAGGC CAAGCGGGTC GGCAAGACCG TCGTCAACTC 8760
8761 CCCGTTGGTG AAGACCGCCG TGCACGGCTG CGACCCCAAC TGGGGCCGGG TCGCCATGGC 8820
8821 GATCGGCAAG TGCTCGGACG ACACCGACAT CGACCAGGAG CGGGTGACGA TCCGCTTCGG 8880
8881 CGAGGTCGAG GTCTATCCGC CGAAGGCCCG GGGCGACCAG GCCGACGACG CGCTGCGGGC 8940
8941 CGCCGTCGCG GAGCATCTGC GGGGCGACGA GGTGGTCATC GGGATCGACC TCGCCATCGC 9000
9001 GGACGGGGCC TTCACCGTCT ACGGCTGCGA CCTCACCGAG GGCTATGTCC GGCTGAACTC 9060
                End of ORF 6-->
9061 GGAGTACACC ACCTGAtccc cggaccaggga acgggccgcc gccccgtlcc ctgtccgctc 9120
9121 ccgtcccgtg tggttatacc gaccgttccc cggctatgcg cacgggacgg agcggccccc 9180
9181 gccgggcccc gccccgccgc acgatgaggg gcgatgccag gtgaccgggg caggagggac 9240
                Beginning of ORF 7-->
9241 ATGGAGACCA CTCGCYCGAC GACTGCCGAC GAGGGCYTCG ACGCCGGGGT ACGGGGAGTG 9300
9301 GTCGCGCCGA CCGACGCCCC GGGCGGGACG CTGCGGCTGG TCCGCACGGA CGACTTCGAC 9360
9361 TCGCTCGACC CCGGCAACAC GTACTACGCC TACACCTGGA ACTTCCTCCG GCTCATCGGC 9420
9421 CGGACGCTGG TCACCTTCGA CACCGCGCCG GGCAAGGCGG GCCAGCGGCT CGTGCCCGAC 9480
9481 CTCGCCGAGT CGCTGGCCGA GTCCTCCGAG GACGGCCGGG TCTGGACCTA CCGGCTGCGC 9540
9541 GAGGGCCTGC GCTACGAGGA CGGCACGCCG GTCGTCTCGG CGGACATCAA GCACGCCATC 9600
9601 GCCCGCAGCA ACTACGGCAC CGATGTCCTG GGCGCCGGTC CGACCTACTT CCGCCACCTC 9660
9661 CTGGGCACCG AGTACGGCGC CCCCTGGCGC GAGCCGGACG CCCACGGACC GGTGACGCTG 9720
9721 GAGACCCCGG ACGAGCCGAC GCTGGTCTTC CGGCTGCGGG AGCCGTTCGC GGGGATGGAT 9780
9781 CTGCTGCCGA CCATGCCGTC CACCACCCCC GTGCCGCCCG ACCCGGACAC CGGCGCCGAG 9840
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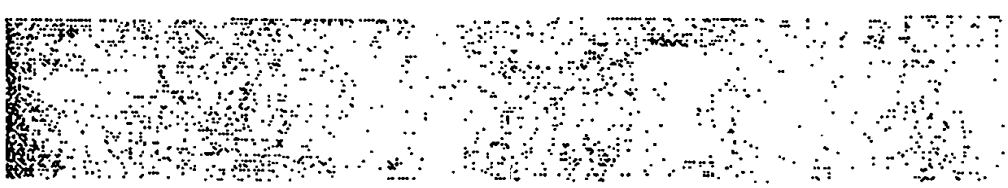

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FIGURE 2 - 7

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9841 TACCGGCTGC GGCCCG7GGC GACCGGCCCG  TACCGGATCG TCTCGTACAC CCGGGGCGAA 9900
9901 CTGGCCGTCC TGGAGCCCAA TCCGCACTCG  GACCCCGAGA CCGACCCCGT GCGCGTCCAG 9960
9961 CGCGCCTCCC GGATCGAGGT GCACCTCGGC  AAGGACCCGC ACGAGGTGGA CCGCATGCTG 10020
10021 CTGGCGGGCG AGGCCCATGT GGACCTCGCG  GGCTTCGGTG TGCAGCCCGC CGCCCAGGAG 10080
10081 CGCATCCTCG CCGAGCCGGA GCTGCGCGCG  CACGCGGACA ACCCGCTGAC CGCCTTCACC 10140
10141 TGGATCTACT GCCTGTCGAG CCGGATCGCC  CCGTTCGACA ATGTGCACTG CCGGCGGGCC 10200
10201 GTGCAGTTCG CCACCGACAA AGCGGCCATG  CAGGAGGCGT ACGCGGCGCC GGTCGGCGGC 10260
10261 GACATCGCGA CCACCCTGCT GCCCCCGACC  CTCGACGCCT ACAAGCACTT CGACGCCTAC 10320
10321 CCGGTCGGCC CCGAGGGCAC CGGCGACCTG  GAGGCCGCCC GCGCCGAGCT GAAGCTGGCC 10380
10381 GGGATGCCCG ACGGCTTCCG CACCAGGATC  GCCGCCCGCA AGGACCGGCT CAAGGAGTAC 10440
10441 CGGGCCGCCG AGGCGCTGGC CGCCGGGCTC  GCCCGGGTCG GCATCGAGGC GGAGGTGCTG 10500
10501 GACTTCCCGT CGGCGGACTA CTTCGACCGC  TACGGCGGCT GCCCGGACTA TCTGCCGCAG 10560
10561 CACGGGATCG GGATCATCAT GTTCGGCTGG  GGCGCCGACT TCCCCGACGG ATACGGCTTC 10620
10621 CTCCAGCAGA TCACCGACGG GCGCGCGATC  AAGGAGCGCG GCAACCAGAA CATGGGCGAG 10680
10681 CTGGACGACC CGGAGATCAA CGCGCTGCTG  GACGAGGGGG CGCAGTGCGC CGACCCGGCG 10740
10741 CGGCGCGCGG AGATCTGGCA CCGCATCGAC  CAGCTCACGA TGGACCACGC GGTCATCGTT 10800
10801 CCGTATCTGT ACCCGCGGTC CCTGCTCTAC  CGGCACCCGG ACACCCGCAA CGCCTTCGTC 10860
                                        End of ORF 7-->
10861 ACCGGCTCCT TCGGGATGTA CGACTACGTG  GCGCTCGGCG CGAAGTGAgc acgggatccg 10920

10921 gcccgcgggc cgtatgtccc ggggccggcc  cccgcccgtt ccccgcccgg tccggtccgg 10980
                    <--End of ORF 6
10981 acccggtcgc ggcccgcTCA GCCGGACATC  CGGGCCCCGG CCGCGACCCC GCGCCGGATC 11040
11041 GGCCAGTGGC CCTGCGCCAG GGGCCGTTCC  ACGCTGCCGC AGGCGAGAGC GGCCTCGCGG 11100
11101 AACTCCGCCT CGTACAGCGC GAGCTGGCGC  AGGAACTGCC GGGTCGGGCC GGTCAGGCTG 11160
11161 GTCCCCCGCG GGCTGCGCAG CAGCAGCCGG  GCGCCGAGGG ACTGCTCCAG CCGGTGAATC 11220
11221 CGGCGGGTGA GCGCCGACTG GCTGATCGAC  AGCACCGCCG CGGCCCGGTT GATGCTGCCG 11280
11281 TGCCGGGCCA CGGCCTGGAG CAGATGCAGA  TCGTCCACAT CCAGTTTGCG GCCCTCGGCC 11340
11341 TGGCCGGGCA CGGAGCCCTG GTCGGGTCCC  GCCCCGAAGC GGCGGGCGTC CGCGCCGGTG 11400
11401 CGCTCCGCGT ACCACTGCGC CCACCAGGGC  TCGTCCAGCA GGTCGCGGTG GTGTTCGGCG 11460
11461 AAGCGCCGGA GCTGGACCTC GGCGATCAGC  GCGGCCAGCC GTCCCGCCAG CGCCCGGGGC 11520
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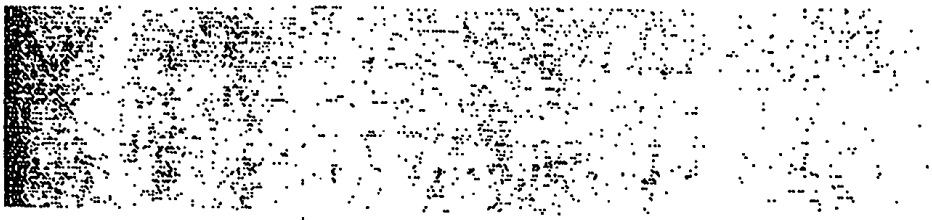

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FIGURE 2 - 0

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11521 ACGATGGTGG GGTCGACGAG CAGACTCGTG GTGCCGCCCC GGCGCTCCGC CAGGGAGCGG 11580
11581 CGCACCAGCG AGGGGTCCTG CACCGCCGGG TGCGTGGGCG AGCCGAGACC TATCGCGTCC 11640
11641 CCGCGGCGCA GGATGCCCCG GGCAACCGAT GCCCCCGTGA TGTGGAGCCG GGTGGGCGCG 11700
11701 GTGAGCCCGG CCAGCTGGAA GACACGTGTC ACCAGGATCT CCGAGCCGGG TCCCCTCTCG 11760
11761 GACACCCAGG TCTCGTCCCG CAGATCGGCG AGCGAGACCT CCCGCCGGGC GGCCAGCGGA 11820
11821 TGGTCCCGGG GCAGGATCAC CCACAGCGGC TCGTCCAGCA CCTCACAGGT GCGCACGGAC 11880
11881 CGCTCCAGGC TGTGCCGGGG GGACTGGAGG CTCCAGGTGT AGGCCGCGTC CACCTGGTAG 11940
11941 CCCGCCAGTT GGGCGGCGAC CTGGTGCGGG GCCTCGTCCC GGACCGACAG CAGCAGGTCC 12000
12001 AGCGAGGCCG CCGCGTCCTC CACCACCTCG TLGAGCAGGG GTTCCGTGGA GACCAGCGAC 12060
12061 AGCACCTCCG GGGCGTCCAC GGCCTCGGAG CCATGGCCGA AGATATGCGT CCGCRCGGCC 12120
12121 AGGTCGACCT GGTGGAAGAA CCGCCGCCCG GCGACCAGGA TGCGGGAGCC CGCGGTGGTC 12180
12181 AGCCGGGCCG TGTGGCGGCT GCGCAGGGTC AGCGGGAGGC CGACGATCCG GTCCAGCCGG 12240
12241 TCGAGTCTGC GCTCCACGGT GCCGTGCCGG ACAGCCGTCC GCCGGGCCAC TTCCATgagg 12300
                                <--beginning of ORF 8
12301 tatccgcagt gtcccaccgc gtccagtaaa gacagatcgc atcggatgac accagcagac 12360
12361 gtcggttctg accccgagag caatgtcggt tcccttttcc gtccanggac( gtaccgctga 12420
12421 attgtccgaa gtggctcttg aattgcttcg gaatcgatcc taggcagcgc cgctcttagg 12480
12481 attctcctcg ccgggaagcg gaacgagccc ggccggatgg cgggcgcgct ccgggccccg 12540
12541 tcccgggaac gggggaccgg gcacggccca gccggccccc cggtccgggc gcgcggcgtg 12600
                                <--end of ORF 9
12601 gacctggtcg gcggacgggt gTCAGACCTG GTCGGTGGGG CGTATGAAGA TCTCGTGGAC 12660
12661 GGTCGCGTGG TGCAGCCGCG TCACGGCGTA GCGGACCGCC TCCGCGATGT CCTGGGCCTG 12720
12721 GAGCTTGCGG ATCTGGCTGA TCCGCTGCTC GTACATCTCC TTGGTGGCGG TGTGGGTGAT 12780
12781 GTGGCCGCGC AGCTCCGTGT CGGTGGTGCC CGGCTCGATG ACGACGACCC GCACCCCGCG 12840
12841 CTCGGTGACC TCCTGGGCCA GCGTCTCGCT GAACGCGTTC ACACCGAAGT TCGTGGCCTG 12900
12901 GTAGACGGCC GCGTTGCGGA CGTTCACCCG GCCCGCGATG GAGGACATCT GCACGACGGT 12960
12961 GCCCTTGGTG CGCAGCAGAT GGGGAAGGGC CGCCCCGGGTC ATGTACATCA GGCCCAGGAG 13020
13021 ATTGGTGTCG ATCATCCGGG TCCAGTCGGT GGTGTCGGCG TCCTCCACCG GGCCGACGAC 13080
13081 CATGATCCCG GCGTTGTTGA CGAGGATGTC GAGGCCGCCC AGCGCCTCGA CGGTGGAGGC 13140
13141 GACGGCAGCG TCCACCCCCT GCCGGTCGGC GACGTCGAGT TCGAGGACAT GGACCTTCGC 13200
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FIGURE 2 - 9

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13201 CCCGGCGGCG GTCAGCTCGT CACCCAGGGC GCGCAGCTTC TCGACCTGGC GCGCGGCGAT 13260
13261 GGCCACGGCG GCGCCCTCGG CGGCCAGGGC GCGGGCCGTG GCCTCGCCGA TGCCTGAGCT 13320
                                    <---beginning of ORF 9
13321 CGCGCCCGTG ATGAGCGCGA CTTTCCCCTG GAGTGCGGAT GGCATcattt cctccacatg 13380
13381 gtgctgcgat cgtggtgagc gtatgaagaa ggggtgcgac ctgccgtgcc gggcgcggtt 13440
13441 ccgtacgccg gaccgttgcg gtgggcacgg ccgaccaggt acggatggcc gcagttcccc 13500
13501 ggggagttcc cggggcatgg tgaataccgc ggcgctctcc ga(ggtcttc ggaggacccc 13560
13561 cggggcttca cagggcatca gcggccggag ttctcccgt cccaggcaga cgctatcagc 13620
13621 gtcgcattca ccgtgaatt ccttcggtg gaccgggtta (gactgttc cgccgggtta 13680
13681 tgcgcgccgc cccggcggac cggccaccg ccccgggggct gcggcagatt gggcgccacg 13740
                                    Beginning of ORF 10--->
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13801 GGTGGCACTG GCGTATCCGA TGLACCGGCT CTGCCCGGTC GACCCGCCGC CGCAACTGGC 13860
13861 CGGGCTGCGG TCCCAGAAGG CCGCGAGCCG GGTGACGCTG TGGGACGGCA GCCAGGTGTG 13920
13921 GCTGGTGACC TCGCACGCCG GGGCCCGGGC CGTCCTGGGC GACCGCCGCT TCACCGCGGT 13980
13981 GACGAGCGCG CCCGGCTTCC CGATGCTGAC CCGCACCTCC CAACTGGTGC GCGCCAACCC 14040
14041 GGAGTCGGCG TCGTTCATCC GCATGGACGA CCCGCAGCAC TCCCGGCTGC GCTCGATGCT 14100
14101 CACCCGGGAC TTCCTGGCCC GCCGCGCCGA GGCGCTGCGC CCCGCGGTGC GGGAGCTGCT 14160
14161 GGACGAGATC CTGGGCGGGC TGGTGAAGGG CGAGCGGCCG GTCGACCTGG TCGCCGGACT 14220
14221 GACGATCCCG GTGCCCTCGC OGGTCATCAC CCTGCTCTTC GCCGCCGGTG ACGACCGCCG 14280
14281 GGAGTTCATC GAGGACCGCA GCGCGGTCCT CATCGACCGC GGCTACACCC CGGAGCAGGT 14340
14341 CGCCAAGGCC CGGGACGAAC TCGACGGCTA TCTGCGGGAG CTGGTCGAGG AGCGGATCGA 14400
14401 GAACCCGCGC ACCGACCTGA TCAGCCGGCT CGTCATCGAC CAGGTGCCGC CGGGCATCT 14460
14461 GCGGGTCGAG GAGATGGTCG CGATGTGCCG GCTGCTGCTG GTGGCCGGTC ACGGCACCAC 14520
14521 CACCAGCCAG GCGAGCCTGA GCCTGCTCAG CCTGCTCACC GACCCGGAGC TGGCCGGCG 14580
14581 CCTCACCGAG GACCCGGCCC TGCTGCCCAA GGCGGTCGAG GAGCTGCTGC GCTTCCTACTC 14640
14641 CATCGTGCAG AACGGGCTGG CCCGTGCCGC GGTGGAGGAC GTCCAGCTCG ACGATGTGCT 14700
14701 CATCCGGGCG GGCGAGGCG TGGTGCTGTC GCTGTCGGCG GGCAACCGGG ACGAGACGGT 14760
14761 CTTCCCCGAC CCGGACCGGG TGGACGTGGA CCGCGACGCC CGCCGCCATC TCGCCTTCGG 14820
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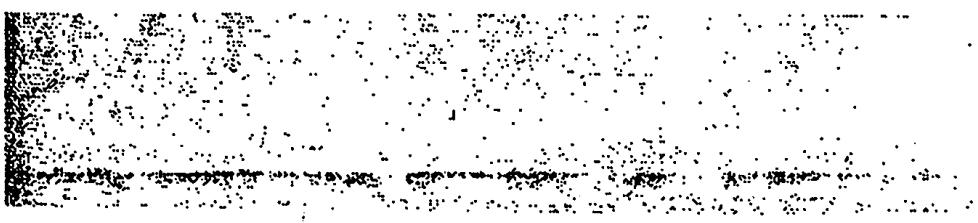

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FIGURE 2 - 10

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15001 gtggagcggc tgaccgtcgt cctcgacgcg  tcggcctgct gcgcgatggg gcgctgcgcg 15060

15061 gccacggccc ccgggatct                                              15079

      |  10     |  20     |  30     |  40     |  50     |  60
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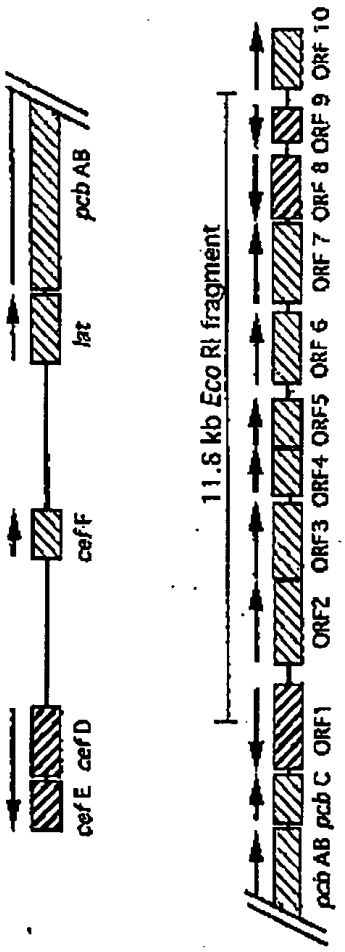
pcb AB
lat
cef F
cef E cef D
11.8 kb Eco RI fragment
pcb AB pcb C ORF1 ORF2 ORF3 ORF4 ORF5 ORF 6 ORF 7 ORF 8 ORF 9 ORF 10
ORF 4 = cda

FIGURE 3

program described above. The AUH sequence had previously been aligned with the three ARC sequences (Szumanski & Hoyle (1990), J. Bacteriol., v. 172, pp. 538-547). Identical matches in two or more sequences are indicated with upper case letters.

Example 2

DNA hybridization

Genomic DNA preparations from various Streptomyces species were isolated as described by Hopwood et al. (1985). For interspecies DNA hybridization analysis, 2.0 µg amounts of genomic DNA preparations were digested with NcoI for 16h, and electrophoresed in 1.0% agarose gels. The separated DNA fragments were then transferred onto nylon membranes (Hybond-N, Amersham) and hybridized with a cla specific probe prepared by labeling an internal 459 bp SalI fragment (Fig. 1) with (α-^{32}P]dATP by nick translation. Hybridization was done as described by Sambrook et al., (1989). Hybridization membranes were washed twice for 30 min in 2X SSC; 0.1% SDS and once for 30 min in 0.1X SSC; 0.1% SDS at 65°C.

Sequences homologous to cla in other Streptomycetes

Three of six producers of β-lactam antibiotics, S. clavuligerus, S. lipmanii and S. jumonjinensis showed positive hybridization signals whereas S. cattleya, S. griseus, and N. lactamdurans did not (data not shown). None of the nonproducing strains examined, S. venezuelae, S. lividans, S. fradiae, S. antibioticus and S. griseofuscus gave any signal. All of the streptomycetes that gave positive signals were producers of clavam-type metabolites (Elson et al., 1987)

Example 3

Disruption of the genomic cla gene

A 2.0 Kb NcoI fragment that contained the entire cla gene was digested at its unique KpnI site and the ends

made blunt by treatment with the Klenow fragment of E. coli DNA polymerase I. A thiostrepton resistance gene (tsr), isolated as a 1085 bp BclI fragment from pIJ702 and cloned into the BamHI site of pUC118 was excised as a SmaI/XbaI fragment and the ends made blunt as above and ligated into the KpnI site of cla. The ligation mixture was introduced into E. coli MV1193 and the transformants screened for the presence of the tsr gene by colony hybridization (Sambrook et al., 1989).

Replacement of the chromosomal cla gene by a copy disrupted by the insertion of tsr, at an internal KpnI site, was achieved by double recombination. Successful gene replacement was apparent when the 2.0 kb NcoI fragment which carries cla in the wild type organism was replaced by a 3.0 Kb NcoI fragment due to the insertion of the 1.0 Kb tsr gene in the mutants. Four of the five mutants tested showed the expected increase in the size of the NcoI fragments, and the larger NcoI fragments also hybridized with a tsr specific probe. The fifth mutant was apparently a spontaneous thiostrepton resistant mutant.

Antibiotic Assay

The agar diffusion assay was used for determining both penicillin/cephamycin and clavulanic acid production. S. clavuligerus strains to be assayed were grown in 10 ml. amounts of Trypticase Soy Broth (TSB; Baltimore Biological Laboratories) medium with 1.0% starch for 48h. The cultures were washed twice with 10.3% sucrose and once with MM (Jensen et al. (1982), J. Antibiot., v. 35, pp. 483-490) and the mycelium resuspended in 10.0 mL of MM. Two millilitres of washed cell suspension was inoculated into 100 mL of MM and incubated at 28°C for 48h. The cultures were harvested by centrifugation, and the supernatants were assayed for both penicillin/cephamycin and clavulanic acid using

bioassay procedures described previously (Jensen et al. (1982), supra).

All of the resulting colonies with disrupted cla genes grew equally well on minimal medium and complex media and produced as much penicillin and cephamycin as did the wild-type, but produced no clavulanic acid (data not shown). HPLC analysis of cell supernatants confirmed the inability of the disrupted cla mutants to synthesize any clavulanic acid (data not shown).

Example 4

Protoplast formation and transformation

E. coli competent cell preparation and transformation were as described by Sambrook et al., (1989). Protoplasts of S. clavuligerus were, prepared, transformed and regenerated as described by Bailey et al. (1984), Bio/Technology, v. 2, pp. 808-811, with the following modifications. Dextrin and arginine in the regeneration medium were replaced by starch and sodium glutamate respectively. Protoplasts were heat shocked at 43°C for 5 min prior to the addition of DNA. Standard procedures were used for protoplasting and transformation of S. lividans (Hopwood et al. (1985)).

The 11.6 kb EcoR1 fragment from K6L2 (Fig. 9) was cloned into the EcoR1 site of pCAT-119. pCAT-119 is derivative of pUC119 which was prepared by insertionally inactivating the ampicillin resistance gene of pUC119 by the insertion of a chloramphenicol acetyltransferase gene (Jensen et al. (1989), Genetics & Molec. Biol. of Ind. Microorg., pp. 239-245 Ed. Hershberger, Amer. Soc. Microbiol). The PCAT-119 plasmid carrying the 11.6 kb fragment was then digested with PstI and ligated to the Streptomyces plasmid pIJ702, which had also been digested with PstI. The resulting bifunctional plasmid carrying the 11.6kb insert was capable of replicating in either E. coli (with selection for chloramphenicol resistance) or in S. lividans (with selection for thiostrepton

resistance). The ligation mixture was transformed to E. coli. Plasmid DNA was isolated from several of the chloramphenicol resistant transformants and analyzed by agarose gel electrophoresis to ensure that the proper plasmid construct was obtained. This isolated plasmid material from E. coli was then transformed into S. lividans as described by Hopwood and transformants were selected by plating onto R2YE medium containing thiostrepton at a concentration of 50 µg/ml.

Thiostrepton resistant S. lividans transformants carrying the bifunctional plasmid with the 11.6 kb insert were patched onto MYM agar plates and allowed to incubate for 48h at 28°C before they were overlayered with molten soft nutrient agar containing penicillin G at a concentration of 1 µg/ml and inoculated with Staphylococcus aureus N-2 as indicator organism (Jensen, 1982). (S. aureus N-2 was obtained from the Department of Microbiology Culture Collection, University of Alberta. Any organism which produces a β-lactamase sensitive to clavulanic acid may be used as indicator organism.) Zones of inhibition which appeared around the S. lividans colonies upon incubation overnight at 30°C were evidence of clavulanic acid production. Clavulanic acid-producing colonies were found amongst these initial S. lividans transformants at a frequency of about 12%. When plasmid DNA was isolated from one of these clavulanic acid-producing transformants and re-introduced into S. lividans, the frequency of clavulanic acid production in these 2nd round transformants was about 40-45%. Figure 6 shows a photograph of an agar plate bearing 2nd. round transformants. Zones of inhibition are seen as clear areas in the agar; these appear on the photograph as dark circular areas.

Example 5

Sequencing of 15 Kb DNA fragment

Ordered sets of deletions were generated as described in Example 3 using fragments of the DNA insert from the cosmid clone K6L2 (Figure 9) and subcloned into the E. coli plasmids pUC118 andpUC119. Overlapping fragments were chosen which extended from the end of the pcbC gene downstream for a distance of about 15 Kb ending at the BglII site. The deletion generated fragments were sequenced in both orientations as described in Example 1. The sequence is shown in Figure 2.

The present invention is not limited to the features of the embodiments described herein, but includes all variations and modifications within the scope of the claims.

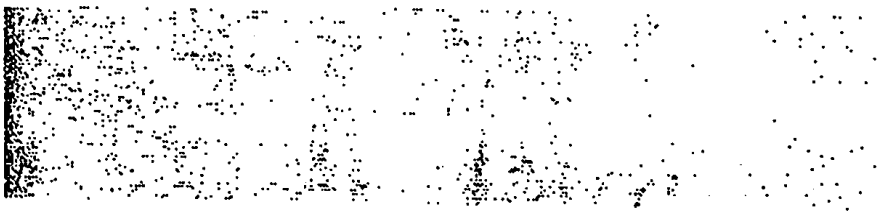

TABLE 1

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2	2216	3937	1722	574
3	3940	5481	1542	514
4	5654	6595	942	314
5	6611	7588	978	326
6	7895	9076	1182	394
7	9241	10 908	1668	556
8*	10 998	12 296	1299	433
9*	12 622	13 365	744	248
10	13 769	14 995	1227	409

* Asterisks denote ORFs which are oriented in the opposite direction.

N-terminal amino acid sequence of CLA	Met	Glu	Arg	Ile	Asp	Ser	His	Val	Ser	Pro	Arg	His	Asp	(Asp)
	Tyr	Arg	Gln	Ile	Pro	Thr	Phe	Met	Arg	(Leu)	Pro			
Potential codons (DNA)	TAT	GCT	CAA	ATT	CCT	ACT	TTT	ATG						
	C	C	G	C	C	C	C							
		A		A	A	A								
		G			G	G								
Probe made - 24-mer oligonucleotide with 8-fold degeneracy	TAC	GCC	CAG	ATC	CCC	ACC	TTC	ATG						
		G			G	G								
Actual DNA sequence	TAC	GCA	CAG	ATC	CCC	ACG	TTC	ATG						

FIGURE 1

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FIGURE 2 - 1

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      1   10        1   20        1   30        1   40        1   50        1   60
    1 gcggaacegg ccgcccctga gcgggggcgga cgggaaggaa acgggccggt cgtcccctcg 60
                                                    (--End of ORF \
   61 ggaggggggc gccggcccgt ccggtgcgcg cggtgggtgc ggcgcgggTC ACCCGGCCGC 120
  121 GAGGTTGCTG AGGAACTTCG CGGCGACGGG GCCCGCGTCG GCGCCGCCCG ACCCGCCGTC 180
  181 CTCCAGCAGG ACCGACCAGG CGATGTTCCG GTCGCCCTGG TAGCCGATCA TCCAGGCGTG 240
  241 CGTCTTCGGC GGCTTCTCGG TGCCGAACTC GGCGGTACCG GTGTTGGCGT GCGGCTGTCC 300
  301 GCCGAGGCCC CGCAGGGCGT CGCCGGCGCC GTCGGTGACG GTCGAACGCA TCATGGAACG 360
  361 CAGCGAGTCG ACGATGCCCG GGCCCATCCC GGGGGCCTGG TGCGGCTTCT TGACCGCGTC 420
  421 GGGCACCAGC ACGGGCTGGT TGAACTCGCC GTGCTTGACG GTGGCGGCGA TGGAGGCCAT 480
  481 CACCAGGGGC GACGCCTCGA CCCTGGCCTG TCCGATGGTC GACGCGGCCT TGTCGTTCTC 540
  541 GCTGTTGGAG ACGGGGACGC TGCCCTCGAA GGTGGAGGCG CCGACGTCCC AGGTGCCGCC 600
  601 GATGCCGAAG GCTTCGGCGG CCTGCTTCAG GCTGCACTCG GAGAGCTTGC TGCGGGAGTT 660
  661 CACGAAGAAC GTGTTGCAGG AGTGGGCGAA GCTGTCCCGG AAGGTCGAGC CCGCGGGCAG 720
  721 CGTGAACTGG TCCTGGTTCT CGAAGCTCTG GCCGTTGACA TGGGCGAACT TCGGGCAGTC 780
  781 GGCCCGCTCC TCCGGGTTCA TCCCCTGCTG GAGCAGGGCC GCGGTGGTGA CCACCTTGAA 840
  841 GGTGGAGCCG GGCGGGTAGC GGCCCTCCAG CGCGCGGTTC ATGCCGGAGG GCACGTTCGC 900
  901 GGCGGCCAGG ATGTTGCCGG TGGCGGGGTC GACGGCGACG ATCGCCGCGT TCTTCTTCGA 960
  961 GCCCTCCAGG GCCGCCGCGG CGGCGGACTG GACCCGCGGG TCGATGGTGG TCTTCACCGG 1020
 1021 CTTGCCCTCG GTGTCCTTGA GGCCGGTGAG CTTCTTGACC ACCTGGCCGC ACTCACGGTC 1080
 1081 CAGGATCACG ACCGAGCGCG CCGCGCCGGA GCCGCCGGTG AGCTGCTTGT CGTAGCGGGA 1140
 1141 CTGGAGGCCC GCCGAGCCCT TGCCGGTCCT GGGGTCGACC GCGCCGATGA TGGAGGCGGC 1200
 1201 CTGGAGGACA TTGCCGTTGG CGTCGAGGAT GTCCGCGCGC TCCCGCGACT TGAGGGCGAG 1260
 1261 GGTCTGCCCC GGAACGATGT GCGGATGGAT CATCTCGGTG TTGAACGCGA CCTTCCACTC 1320
 1321 CTTGCCGCCG CCGACGACCT TCGCGGTGGA GTCCCAGGCG TACTCCTCGG CCCCGGGGAG 1380
 1381 GGTCATTCTG ACGGTGAACG GTATCTCCAC CTCGCCCTCG GGGTTCTTCT CCCCGGTCTT 1440
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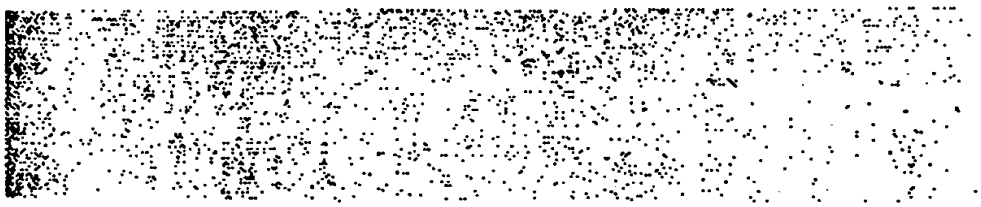

FIGURE 2 - 2

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1861 gaggcccgtt cccacccctt gggggggagc  ggcacccgga aggtgaccga ggcaacatcc 1920
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1981 gtgacagcgg ggugtagcga caaaacggtc  agacccctga agggaattna ctgaattcga 2040
2041 gtcatcgggt tcggcgacgg atgggcggtt  cggccacgca ccgtcactct tcgtcccctc 2100
2101 ttcacaagaa ctcccgatac gtggagaaga  gagcgtgaag agcgcgtccg gtcagggtig 2160
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                                                                Begin-
                ning of ORF 2--->
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2341 CTTCGACGAG GTCGACCCCA TCGACTTCGT  TCYGACCCGC CACGAGTTCA CCGCGGGTGT 2400
2401 CGCCGCTGAT GTCCTCGCGC GGATCACCGG  TCGCCCCCAG GCGTGCTGGG CCACCCTGRG 2460
2461 CCCCGGTATG ACCAACCTCT CCACCGGTAT  CGCCACGTCC GTCCTGGACC GCTCGCCGGT 2520
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2581 CCTGGACTCG GTGGCGATCG TCGCCCCGAT  GTCCTTGTAC GCCGTGGAGC TCCAGCGGCC 2640
2641 CCACGAGATC ACCGACCTCG TCGACTCCGC  CGTGAACGCG GCCATGACCG AGCCGGTCGG 2700
2701 GCCCTCCTTC ATCTCCCTCC CGGTGGACCT  GCTCGGCTCC TCCGAGGGCA TCGACACCAC 2760
2761 CGTCCCCAAC CCGCCGGCGA ACACCCCGGC  GAAACCGGTC GGCGTCGTCG CCGACGGCTG 2820
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3001 GCTGAACTAC GGCGCCGTCA CCGGCTACAT  GGACGGCATC CTCAACTTCC CGGCGCTCCA 3060
3061 GACCATGTTC GCCCCGGTGG ACCTCGTCCT  CACCGTCGGC TACGACTACG CCGAGGACCT 3120
3121 GCGCCCGTCC ATGTGGCAGA AGGGCATCGA  GAAGAAGACC GTCCGTATCT CCCCGACGGT 3180
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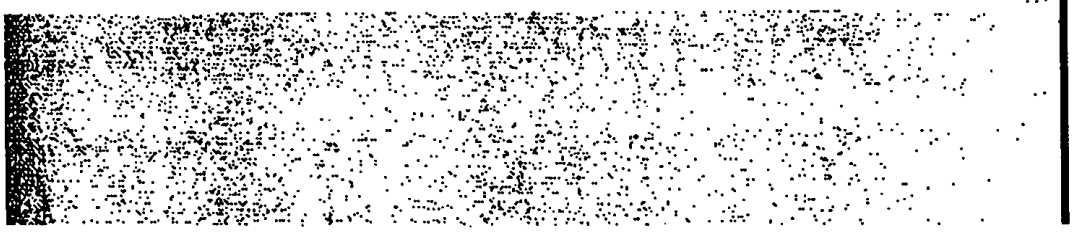

FIGURE 2 - 3

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3421 CGAGGGCACG ATCGTCTCCG ACATCGGCTT  CTTCGGTCAC TACCGTCTGC TCTTCGCCCG 3480
3481 CGCCGACCAG CCCTTCGGCT TCCTCACCTC  GGCGGGCTGC TCCAGCTTCG GCTACGGCAT 3540
3541 CCCCGCCGCC ATCGGCGCCC AGATGGCCCG  CCCGGACCAG CCGACCTTCC TCATCGCGGG 3600
3601 TGACGGCGGC TTCCAGTTCA ACATCTCCGA  CCTGGAGACC ATCGCCCGGC TCAACCTGCC 3660
3661 GATCGTGACC GTCGTCGTCA ACAACGACAC  CAACGGCCTG ATCGAGCTGT ACCAGAACAT 3720
3721 CGGTCACCAC CGCAGCCACG ACCTGGCGGT  CAAGTTCGGC GGCGTCGACT TCGTCGCGCT 3780
3781 CGCCGAGCCC AACGGTGTCG ACGCCACCCG  CGCCACCAAC CGCGAGGAGC TGCTCGCGGC 3840
3841 CCTGCGCAAG GGTGCCGAGC TGGGTCGTCC  GTTCCTCATC GAGGTCCTGG TCAAGTACGA 3900
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4021 TTCGCGACCC GGGGCAGCCA CACCGACATC  GACACGCCCC AGGGGGAGCG CTCGCTCGCG 4080
4081 GCGACCCTGG TGCACGCCCC CTCGGTCGCG  CCCGACCGCG CGGTGGCGCG CTCCCTCACC 4140
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4201 GTGCTGCCCG CCGGACCCGC GCCGGAGGGG  GACGCGGAGC TGGTCCTGCG GCTGCTGGAA 4260
4261 CGCTATGACC TGCATGCCTT CCGGCTGGTG  AACGGGCGCT TCGCGACCGT GGTGCGGACC 4320
4321 GGGGACCGGG TCCTGCTCGC CACCGACCAC  GCCGGTTCGC TGCCGCTGTA CACCTGTGTG 4380
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4441 AAGGCCTTCC CGCTCGCGGA CGCCCGCCGG  GTCGCCGGTC TGACCGGTGT CTACCAGGTG 4500
4501 CCCGCGGGCG CCGTGATGGA CATCGACCTC  GGCTCGGGCA CCGCCGTCAC CGACCGCACC 4560
4561 TGGACCCCGG GCCYCTCCCG CCGCATCCTG  CCGGAGGGCG AGGCCGTCGC GGCCGTGCGG 4620
4621 GCCGCGCTGG AGAAGGCCGT CGCCCAGCGG  GTCACCCCCG GCGACACCCC GTTGGTGGTG 4680
4681 CTCTCCGGCG GAATCGACTC CTCCGGGGTC  GCGGCCTGTG CGCACCGGGC GGCCGGGGAA 4740
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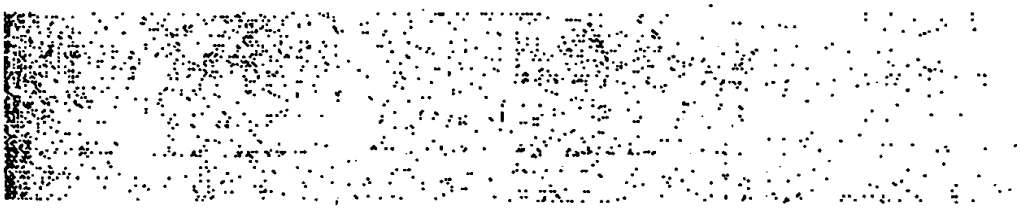

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FIGURE 2 - 4

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5221 GCCGCGATGG CCGACGCCCT CCCGGCGGAG  ACCGTCAAGC GGCCCAAGCT GGGCGTCCAC 5280
5281 GAGGGCTCGG GCACGACGTC CTCGTTCTCC  CGGCTGCTGC TGGACGACGG TGTCGCCGAG 5340
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6181 GGCGATGGTC CAGATCGGCA TCCGGGGCCA  CAACCCGAAG CCGGACTCGC TCGACTACGC 6240
6241 CCGGGGCCAC GGCGTCCGGG TGGTCACGGC  GGACGAGTTC GCCGAGCTGG GGGTGGGCGG 6300
6301 GACCGCCGAC CTGATCGGCG AGAAGGTCGG  CCAGCGGCCC GTGTACGTCT CGGTCGACAT 6360
6361 CGACGTGGTC GACCCCGCCT TCGCCCCCGG  TACGGGCACG CCCGCGCCGG GCGGGCTCCT 6420
6421 CTCGCGCGAG GTGCTGGCGC TGCTGCGCTG  CGTGGGTGAC CTCAAGCCGG TCGCCTTCGA 6480
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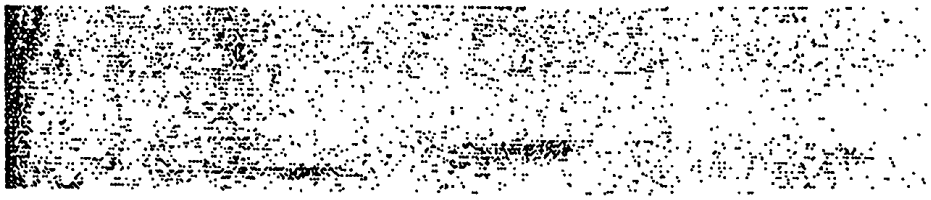

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FIGURE 2 - 5

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                                                                End of ORF 4-->
6541 GATCGGTGCG GAACTGCTCT ACCABTACGC CCGABCCCAC AGAACCCAGT TGTGAaggag 6600
                Beginning of ORF 5-->
6601 ccatcgtgtc ATCGCCTCTC CGATAGTTGA CTGCACCCCG TACCGCGACG AGCTGCTCGC 6660
6661 GCTCGCCTCC GAGCTTCCCG AGGTGCCGCG CGCGGACCTC CATGGCTTCC TCGACCAGGC 6720
6721 GAAGACGCTG GCCGCCCGTC TCCCCGACGG GCTGGCCGCC GCTCTCGACA CCTTCAACGC 6780
6781 CGTGGGCAGC GAGGACGGTT ATCTGCTGCT GCGCGGGCTG CCCGTCGACG ACAGCGAGCT 6840
6841 GCCCGAGACG CCGACCTCCA CCCCGCCCCC GCTGGACCGC AAGCCGCTGG TGATGGAGGC 6900
6901 CATGCTCGCG CTGGCCGGCC GCGGGCTCCG TCTGCACACG GGGTACCAGG AGCTGCGCTC 6960
6961 GGGCACGGTC TACCACGACG TGTACCCGTC GCCCGGCGCG CACTACCTGT CCTCGGAGAC 7020
7021 CTCCGAGACG CTGCTGGAGT TCCACACCGA GATGGCGTAC CACATCCTCC AGCCGAACTA 7080
7081 CGTCATGCTG GCCTGCTCCC GCGCGGACCA CGAGAACCGG CCGGAGACGC TGGTCCGCTC 7140
7141 CGTCCCCAAG GCGCTGCCCC TGCTGGACGA CAAGACCCGG GCCCGTCTCT TCGACCGCAA 7200
7201 GGTGCCCTGC TGCGTGGACG TGGCCTTCCG CGGCGGGGTC GACGACCCGG GCGCGATCGC 7260
7261 CAACGTCAAG CCGCTCTACG GGGACGCGAA CGACCCGTTC CTCGGGTACG ACCGCGAGCT 7320
7321 GCTGGCGCCG GAGGACCCCG CGGACAAGGA GGCCGTCGCC CATCTGTCCC AGGCGCTCGA 7380
7381 CGATGTGACC GTCGGGGTGA AGCTCGTCCC CGGTGACGTC CTCATCATCG ACAACTTCCG 7440
7441 CACCACGCAC GCGCGGACGC CGTTCTCGCC CCGCTGGGAC GGGAAGGACC GCTGGCTGCA 7500
7501 CCGCGTCTAC ATCCGCACCG ACCGCAATGG ACAGCTCTCC GGCGGCGAGC GCGCGGGCGA 7560
                End of ORF 5-->
7561 CACCATCTCG TTCTCGCCGC GCCGCTGAgc cgggctcccc gaggcccatgg gcccccggcgc 7620
7621 cggaacggc tcccgtcct gccccatcac ccgccgcgcg ggtgagggggg cgggcccctt 7680
7681 tgtgccgggt gcgtgcgtc ctgcgagggt gccggggcgg ggcggacggc ggaggtgccc 7740
7741 ggcggccggg tgccgtgcgc cgcccgtggg tgctgtacag cactccgtgt gccgtgcgcc 7800
7801 acccccgtgcc laaatttgcc actctatggg aaatcaalgca gagtgcgacg ggtgaggccg 7860
                                                Beginning of ORF 6-->
7861 tcgccgtgcc ctttccgtga cggggagcgc tgacATGTCC GACAGCACAC CGAAGACGCC 7920
7921 CCGGCGATTC GTGGTGCACA CGGCGCCCGT GGGCCTGGCC GACGACGCCC GTCACGACTT 7980
7981 CACCGTCCTC GCCTCCATCG CCCCGGCCAC CGTGAGCGCC GTCTTCACCC GCTCCCGCTT 8040
8041 CGCCGGCCCG AGCGTCGTGC TGTGCCGGGA GGCGGTGCCC GACGGGCAGG CGCGCGGTGT 8100
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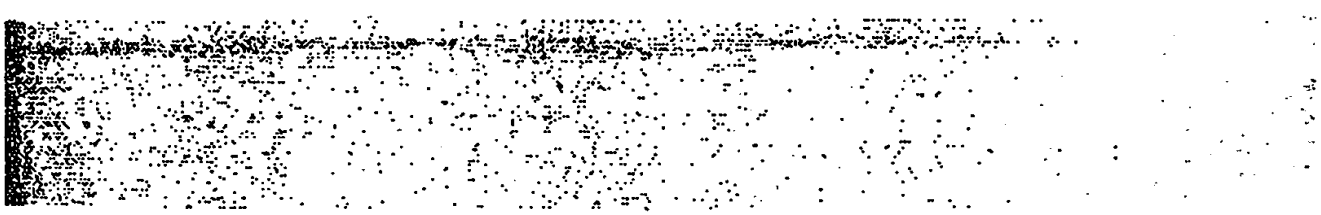

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FIGURE 2 - 5

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6661 GCTCGCCTCC GAGCTTCCCG AGGTGCCGCG CGCGGACCTC CATGGCTTCC TCGACCAGGC 6720

6721 CAAGACGCTG GCCGCCCGTC TCCCCCAGGC GCTGGCCGCC GCTCTCGACA CCTTCAACGC 6780

6781 CGTGGGCAGC CAGCACGGTT ATCTGCTGCT GCGCGGGCTG CCCGTCGACG ACAGCGAGCT 6840

6841 GCCCGAGACG CCGACCTCCA CCCCGCCCCC GCTGGACCGC AAGCCGCTGG TGATGGAGGC 6900

6901 CATGCTCGCG CTGGCCGGCC GCCGGCTCGC TCTGCACACG GGGTACCAGG AGCTGCGCTC 6960

6961 GGGCACGGTC TACCACGACG TGTACCCGTC GCCCGGCGCC CACTACCTGT CCTCGGAGAC 7020

7021 CTCCGAGACG CTGCTGGAGT TCCACACCGA GATGGCGTAC CACATCCTCC AGCCGAACTA 7080

7081 CGTCATGCTG GCCTGCTCCC GCGCGGACCA CGAGAACCGG GCGGAGACGC TGGTCGGCTC 7140

7141 GGTCCGCAAG GCGCTGCCGC TGCTGGACGA CAAGACCCGG GCCCGTCTCT TCGACCGCAA 7200

7201 GGTGCCCTGC TGCGTGGACG TGGCCTTCGG CGGCGGGGTC GACGACCCGG GCGCGATCGC 7260

7261 CAACGTCAAG CCGCTGTACG GGGACGCGAA CGACCCGTTC CTCGGGTACG ACCGCGAGCT 7320

7321 GCTGGCGCCG GAGGACCCCG CGGACAAGGA GGCCGTCGCC CATCTGTCCC AGGCGCTCGA 7380

7381 CGATGTGACC GTCGGGGTGA AGCTCGTCCC CGGTGACGTC CTCATCATCG ACAACTTCCG 7440

7441 CACCACGCAC GCGCGGACGC CGTTCTCGCC CCGCTGGGAC GGGAAGGACC GCTGGCTGCA 7500

7501 CCGCGTCTAC ATCCGCACCG ACCGCAATGG ACAGCTCTCC GCCGCGCAGC GCGCGGCGA 7560
                End of ORF 5-->
7561 CACCATCTCG TTCTCGCCGC GCCGCTGAgc ccggctcccc gaggccctgg gccccggcgc 7620

7621 cggcaacggc tcccggtcct gccccatcac ccgccgcgcg ggtgaggggg cggccccctt 7680

7681 tgtgccgggt gccgtgcgtc ctgcgagggt gccggggcgg ggcggacggc ggaggtgccc 7740

7741 ggcggccggg tgccgtgcgc cgcccgtggg tgctgtacag cactccgtgt gccgtgcgcc 7800

7801 acccccgtgc laaattigcc actctatggg aaatcatgca gagtgcgacg ggtgaggccg 7860
                                                Beginning of ORF 6-->
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7921 CCGGGGATTC GTGGTGCACA CGGCGCCGGT GGGCCTGGCC GACGACGGCC GCCACGACTT 7980

7981 CACCGTCCTC GCCTCCACCG CCCCGGCCAC CGTGAGCGCC GTCTTCACCC GCTCCCGCTT 8040

8041 CGCCGGCCCG AGCGTCGTGC TGTGCCGGGA GGCGGTGCCC GACGGGCAGG CGCGCGGTGT 8100

8101 GGTGGTCCTG GCCCGCAACG CGAATGTCGC GACCGGCCTG GAGGCCCAGG AGAACGCGCG 8160
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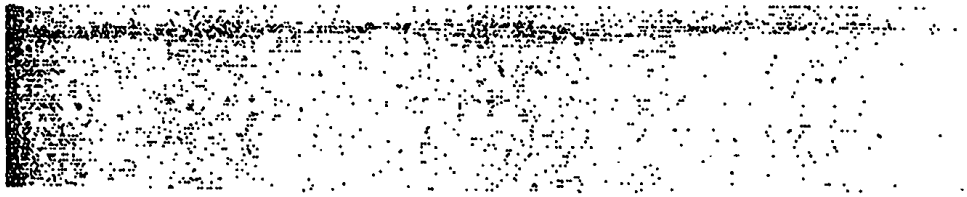

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FIGURE 2 - 6

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8281 GCTGGAGTGG CCCGCCGGGG ACGGCCGGTT CGACCGCGCG GCCCGCGCCA TCATGACGAC 8340

8341 CGACACCCCC CCCAAGGAGG TCCGGGTCAG CGTCGGCGGG GCGACCCTCG TGGGCATCGC 8400

8401 CAAGGGCGTC GGCATGCTGG AGCCCGACAT GGCGACGCTG CTGACCTTCT TCGCCACGGA 8460

8461 CGCCCGGCTG GACCCGGCCG AGCAGGACCG CCTCTTCCGC CGGGTCATGG ACCGCACCTT 8520

8521 CAACGCGGTC AGCATCGACA CCGACACCTC CACCAGCGAC ACGGCGGTGC TGTTCGCCAA 8580

8581 CCGCCTGGCG GGCGAGGTCG ACGCCGGGGA GTTCGAGGAG GCGCTGCACA CGGCGCGCCT 8640

8641 GGCCCTGGTC AAGGACATCG CGAGCCACGG CGAGGGCGCG GCCAAGCTGA TCGAGGTCCA 8700

8701 GGTCACCGGC CCCCGCGACG ACGCCCAGGC CAAGCGGGTC GGCAAGACCG TCGTCAACTC 8760

8761 CCCGTTGGTG AAGACCGCCG TGCACGGCTG CGACCCCAAC TGGGGCCGGG TCGCCATGGC 8820

8821 GATCGGCAAG TGCTCGGACG ACACCGACAT CGACCAGGAG CGGGTGACGA TCCGCTTCGG 8880

8881 CGAGGTCGAG GTCTATCCGC CGAAGGCCCG GGGCGACCAG GCCGACGACG CGCTGCGGGC 8940

8941 CGCCGTCGCG GAGCATCTGC GGGCGACGA GGTGGTCATC GGGATCGACC TCGCCATCGC 9000

9001 GGACGGGGCC TTCACCGTCT ACGGCTGCGA CCTCACCGAG GGCTATGTCC GGCTGAACTC 9060
End of ORF 6-->

9061 GGAGTACACC ACCTGatccc cggacagggg acgggccgcc gcccccgtcc atgtccgatc 9120

9121 ccgtcccgtg tggttatacc gaccgttccc cggctatgcg cacgggccgg agcggccccc 9180

9181 gccgggcccc gcccggccgc acgatgaggg gcgatgccag gtgacgaggg caggagggac 9240
Beginning of ORF 7-->

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9361 TCGCTCGACC CCGCAACAC GTACTACGCC TACACCTGGA ACTTCCTCCG GCTCATCGGC 9420

9421 CGGACGCTGG TCACCTTCGA CACCGCGCCG GGCAAGGCGG GCCAGCGGCT CGTGCCCGAC 9480

9481 CTCGCCGAGT CGCTGGGCGA GTCCTCCGAG GACGGCCGGG TCTGGACCTA CCGGCTGCGC 9540

9541 GAGGGCGTGC GCTACGAGGA CGGCACGCCG GTCGTCTCGG CCGACATCAA GCACGCCATC 9600

9601 GCCCGCAGCA ACTACGGCAC CGATGTCCTC GGCGCCGGTC CGACCTACTT CCGCCACCTC 9660

9661 CTGGGCACCG AGTACGGCGC CCCCTGGCGC GAGCCGGACG CCGACGGACC GGTGATGCTG 9720

9721 GAGACCCCGG ACGAGCCGAC GCTGGTCTTC CGGCTGCGGC AGCCGTTCGC GGGGATGGAT 9780

9781 CTGCTGCCGA CCATGCCGTC CACCACCCCC GTGCCGCGCG ACCCGGACAC CGGCGCCGAG 9840

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FIGURE 2 - 7

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9841 TACCGGCTGC GGCCCG?GGC GACCGGCCCG  TACCGGATCG TCTCGTACAC CCGCGGCCAA 9900
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9961 CGCGCCTCCC GGATCGAGGT GCACCTCGGC  AAGGACCCGC ACGAGGTGGA CCGCATGCTG 10020
10021 CTGGCGGGCG AGGCCCATGT GGACCTCGCG  GGCTTCGGTG TGCAGCCCGC CGCCCAGGAG 10080
10081 CGCATCCTCG CCGAGCCGGA GCTGCGCGCG  CACGCGGACA ACCCGCTGAC CGGCTTCACC 10140
10141 TGGATCTACT GCCTGTCGAG CCGGATCGCC  CCGTTCGACA ATGTGCACTG CCGGCGGGCC 10200
10201 GTGCAGTTCG CCACCGACAA AGCGGCCATG  CAGGAGGCGT ACGGCGGCGC CGTCGGCGGC 10260
10261 GACATCGCGA CCACCCTGCT GCCCCCGACC  CTCGACGCCT ACAAGCACTT CGACCGCTAC 10320
10321 CCGGTCGGCC GCGAGGCCAC CGGCGACCTG  GAGGCCGCCC GCGCCGAGCT GAAGCTGGCC 10380
10381 GGGATGCCCG ACGGCTTCCG CACCAGGATC  GCCGCCCGCA AGGACCGGCT CAAGGAGTAC 10440
10441 CGGGCCGCCG AGGCGCTGGC CGCCGGGCTC  GCCGGGTCG GCATCGAGGC GGAGGTGCTG 10500
10501 GACTTCCCGT CGGGCGACTA CTTCGACCGC  TACGGCGGCT GCCCCGACTA TCTGCGCGAG 10560
10561 CACGGGATCG GGATCATCAT GTCGGCTGG  GGCGCCGACT TCCCCGACGG ATACGGCTTC 10620
10621 CTCCAGCAGA TCACCGACGG GCGCGCGATC  AAGGAGCGCG GCAACCAGAA CATGGGCGAG 10680
10681 CTGGACGACC CGGAGATCAA CGCGCTGCTG  GAGGAGGGGG CGCAGTGCGC CGACCCGGCG 10740
10741 CGGCGCGCGG AGATCTGGCA CCGCATCGAC  CAGCTCACGA TGGACCACGC GGTCATCGTT 10800
10801 CCGTATCTGT ACCCGCTGGT CCTGCTCTAC  CGGCACCCGG ACACCCGCAA CGCCTTCGTC 10860
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10921 gcccccgggac cgtatgtccc gggccgggac  cccgcccgtt cccagcccgg tccggtccgg 10980
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10981 acccggtcgc ggccccgTCA GCCGGACATC  CGGGCCCCGG CCGCGACCCC GCGCCGGATC 11040
11041 GGCCAGTGGC CCTGCGCCAG GGGCCGTTCC  ACGCTGCGGC AGGCGAGAGC GGCCTCGCGG 11100
11101 AACTCCGCCT CGTACAGCGC GAGCTGGCGC  AGGAACTGCC GGGTCGGGCC GGTCAGGCTG 11160
11161 GTCCCCCGCG GGCTGCGCAG CAGCAGCCGG  GCGCCGAGGG ACTGCTCCAG CCGGTGAATC 11220
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11281 TGCCGGGCCA CGGCCTGGAG CAGATCCAGA  TCGTCCACAT CCAGTTTGCG GCCCTCGGCC 11340
11341 TGGCCGGGCA CGGAGCCCTG GTCGGGTCCC  GCCCCGAAGC GGCGGGCGTC CGCGCCGGTG 11400
11401 CGCTCCGCGT ACCACTGCGC CCACCACGGC  TCGTCCAGCA GGTCGCGGTG GTGTTCGGCG 11460
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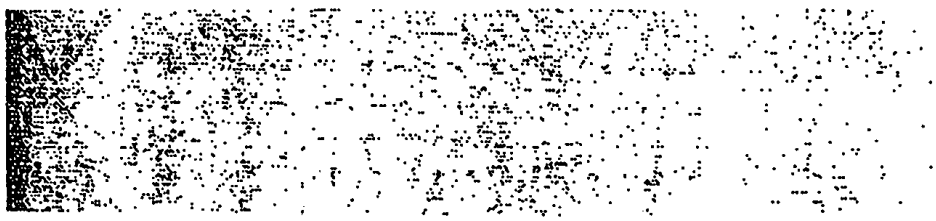

FIGURE 2 - 8

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11641 CCGCGGCGCA GGATGCCCCG GGCAACCGAT GCCCCCGTGA TGTGGAGCCG GGTGGGCGCG 11700
11701 GTGAGCCCGG CCAGCTCGAA GACACGTGTC ACCAGGATC? CCGAGCCGGG TCCCGTCTCG 11760
11761 GACACCCAGG TCTCGTCCCG CAGATCGGCG AGCGAGACCT CCCGCCGGGC GGCCAGCGGA 11820
11821 TGGTCCCGGG GCAGGATCAC CCACAGCGGG TCGTCCAGCA CCTCACAGGT GCGCACGGAC 11880
11881 CGCTCCAGGC TGTGCCGGGG GGACTGGAGG CTCCAGGTGT AGCCCGCGTC CACCTGGTAG 11940
11941 CCCGCCAGTT GGGCGGCGAC CTGGTGCGGG GCCTCGTGCC GGACCGACAG CAGCAGGTCC 12000
12001 AGCGAGGCCG CCGCGTCCTC CACCACCTCG TCGAGCAGGG GTTCCGTGGA GACCAGCGAC 12060
12061 AGCACCTCCG GGGCGTCCAC GGCCTTGGAG CCATGGCCGA AGATATGCGT CCGCRCGGCC 12120
12121 AGGTCGACCT GGTGGAAGAA CCGCCGCCCG GCGACCAGGA TGCGGGAGCC CGCGGTGGTC 12180
12181 AGCCGGGCCG TGTGGCGGCT GCGCAGGGTC AGCGGGAGGC CGACGATCCG GTCCAGCCGG 12240
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12241 TCGAGTCTGC GCTCCACGGT GCCGTGCCGG ACACCCGTCC GCCGGGCCAC TTCCATgagg 12300
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12361 gtcggttctg acccgagaga caatgtcggt tcccttttcc gtcaaggact gtacagctga 12420
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12481 attctcctcg ccgggaagcg gaacgagccc ggccggatgg cgggcgcgct ccgggcgccg 12540
12541 tcccgggaac gggggacggg gcacggcacg gccggccacc cggtccgggc gcgcggcgtg 12600
                                <--end of ORF 9
12601 gacctggtcg gcggacgggt gTCAGACCTG GTCGGTGGGG CGTATGAAGA TCTCGTGGAC 12660
12661 GGTCGCGTGG TGCGGCCGCG TCACGGCGTA GCGGACCGCC TCCGCGATGT CCTGGGCCTG 12720
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12841 CTCGGTGACC TCCTGGGCGA GCGTCTCGCT GAACGCGTTC ACACCGAACT TCGTGGCCTG 12900
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13081 CATGATCCCG GCGTTGTTGA CGACGATGTC GAGGCCGCCC AGCGCCTCGA CGGTGGAGGC 13140
13141 GACGGCGGCG TCCACCCCCT GCCCGTCGGC GACGTCGAGT TCGAGGACAT GGACCTTCGC 13200
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FIGURE 2 - 9

```
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13321 CGCGCCCGTG ATGAGCGCGA CTTTCCCCTG GAGTGCGGAT GGCATcatti ccticcacatg 13380
13381 gtgctgcgat cgtggtgagc gtatgaagaa ggggtgagac ctgccgtgcc gggcgcgggtt 13440
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13861 CGGGCTGCGG TCCCAGAAGG CCGCGAGCCG GGTGACGCTG TGGGACGGCA GCCAGGTGTG 13920
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13981 GACGAGCGCG CCCGGCTTCC CGATGCTGAC CCGCACCTCC CAACTGGTGC CCGCCAACCC 14040
14041 GGAGTCGGCG TCGTTCATCC GCATGGACGA CCCGCAGCAC TCCCGGCTGC GCTCGATGCT 14100
14101 CACCCGGGAC TTCCTGGCCC GCCGCGCCGA GGCGCTGCGC CCCGCGGTGC GGGAGCTGCT 14160
14161 GGACGAGATC CTGGGCGGGC TGGTGAAGGG CGAGCGGCCG GTCGACCTGG TCGCCGGACT 14220
14221 GACGATCCCG GTGCCCTCGC GGGTCATCAC CCTGCTCTTC GGCGCCGGTG ACGACCGCCG 14280
14281 GGAGTTCATC GAGGACCGCA GCGCGGTCCT CATCGACCGC GGCTACACCC CGGAGCAGGT 14340
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14701 CATCCGGGCG GGCCAGGGCG TGGTGCTGTC GCTGTCGGCG GGCAACCGGG ACGAGACGGT 14760
14761 CTTCCCCGAC CCGGACCGGG TGGACGTGGA CCGCGACGCC CGCCGCCATC TCGCCTTCGG 14820
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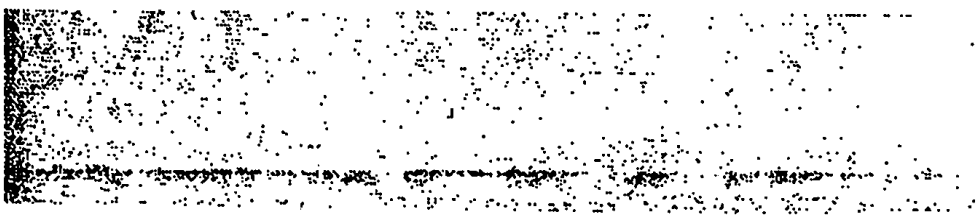

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FIGURE 2 - 10

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15001 gtggagcggc tgaccgtcgt cctcgacgcg  tcggcctgct gcgcgatggg gcgctgcgcg 15060

15061 gccacggccc ccgaggatct                                             15079

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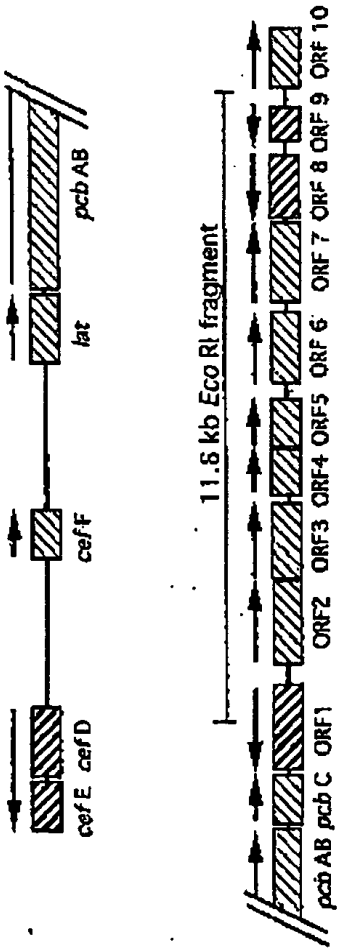
pcb AB
lat
cef F
cef E cef D
11.6 kb Eco RI fragment
pcb AB pcb C ORF1 ORF2 ORF3 ORF4 ORF5 ORF 6 ORF 7 ORF 8 ORF 9 ORF 10
ORF 4 = cla

FIGURE 3

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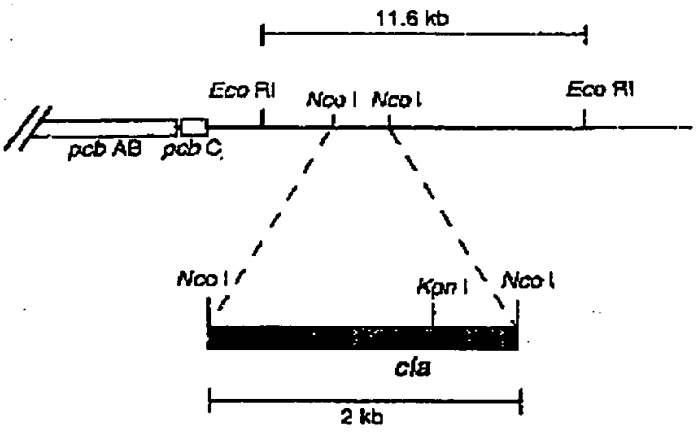

FIGURE 4

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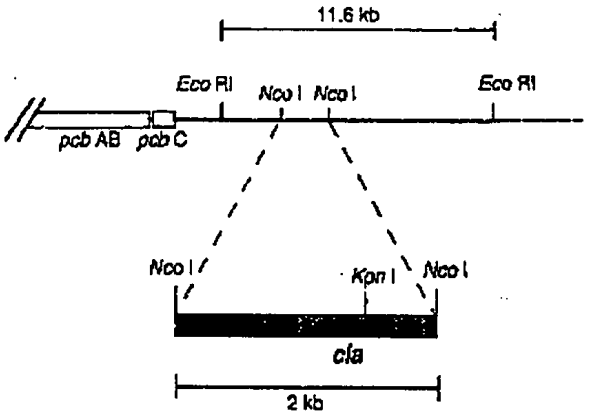


FIGURE 4

2108113

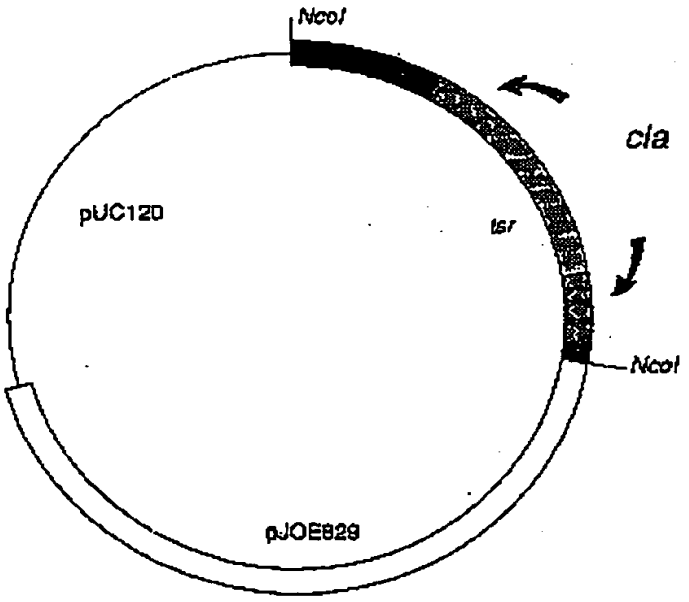


Figure 5

2108113

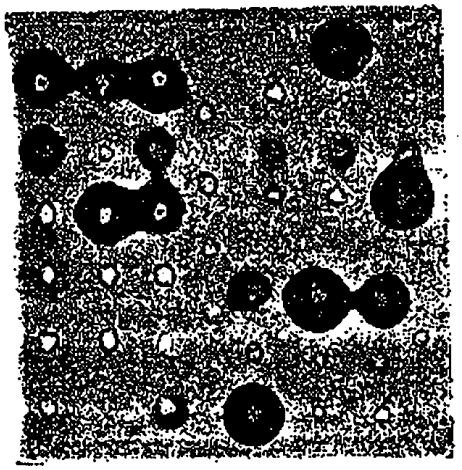


FIGURE 6

2103113

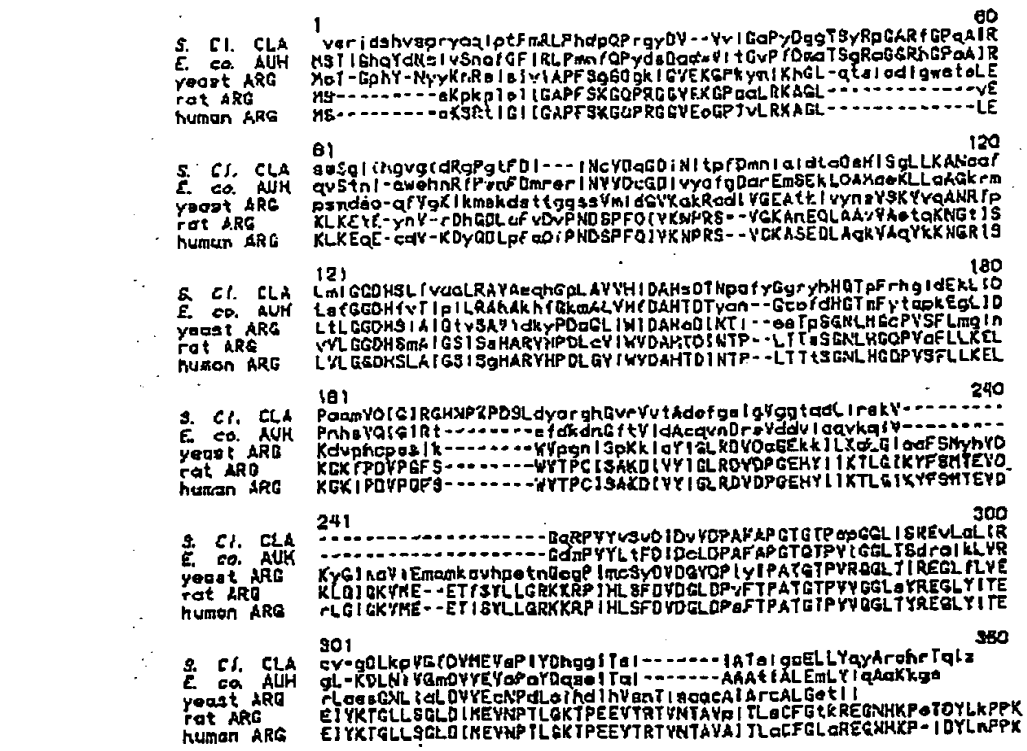


FIGURE 7

2108113

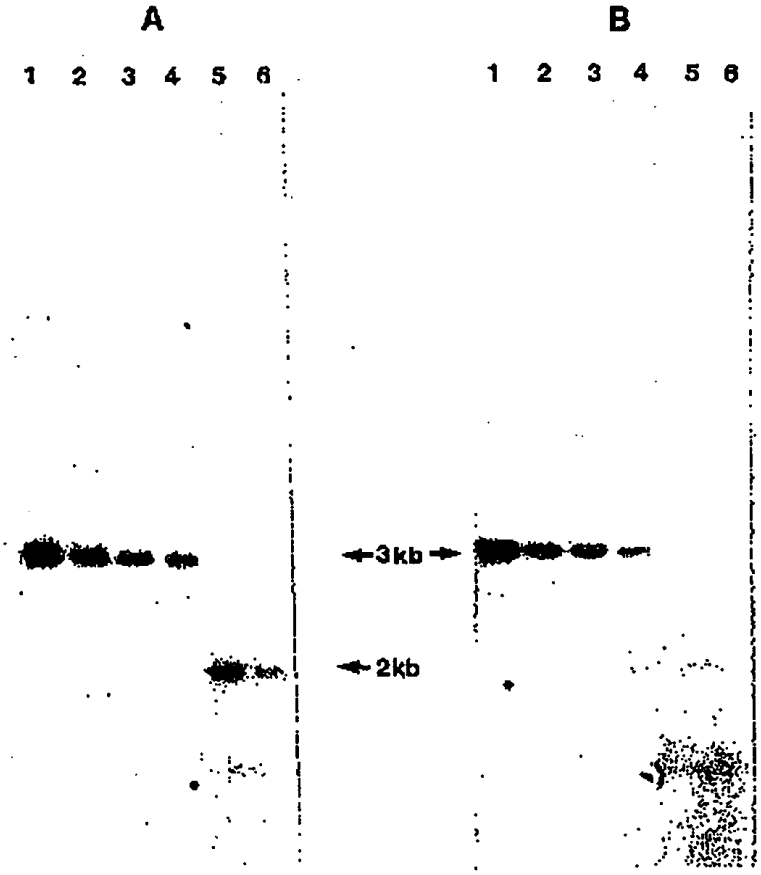


FIGURE 8

2108113

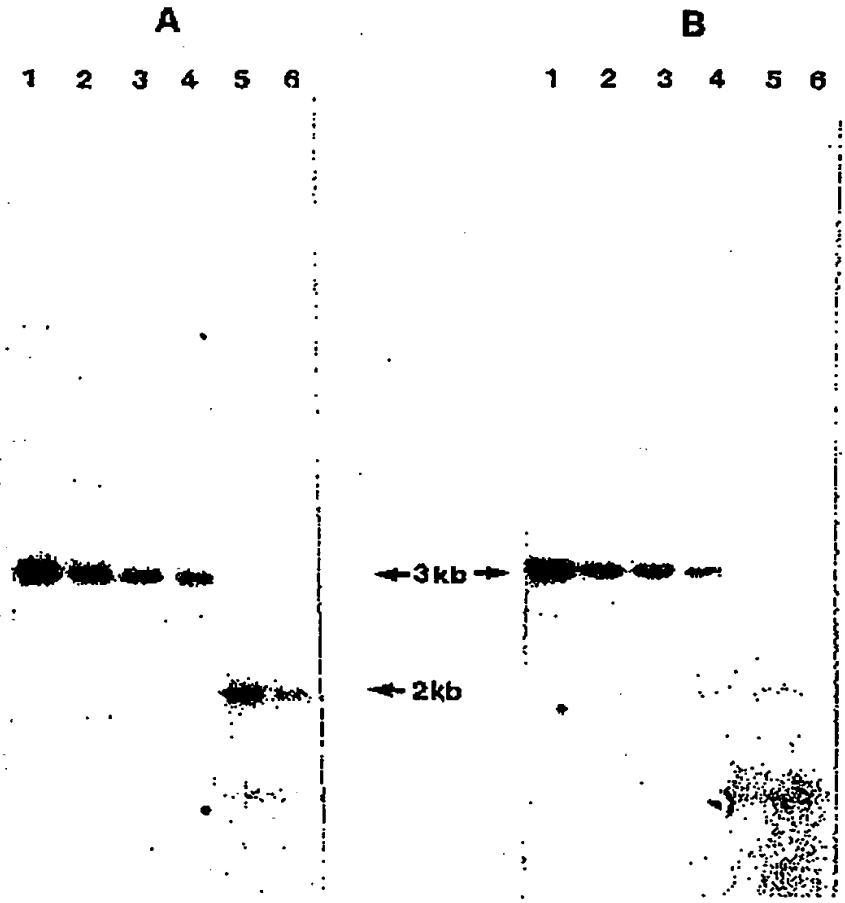


FIGURE 8

2108113

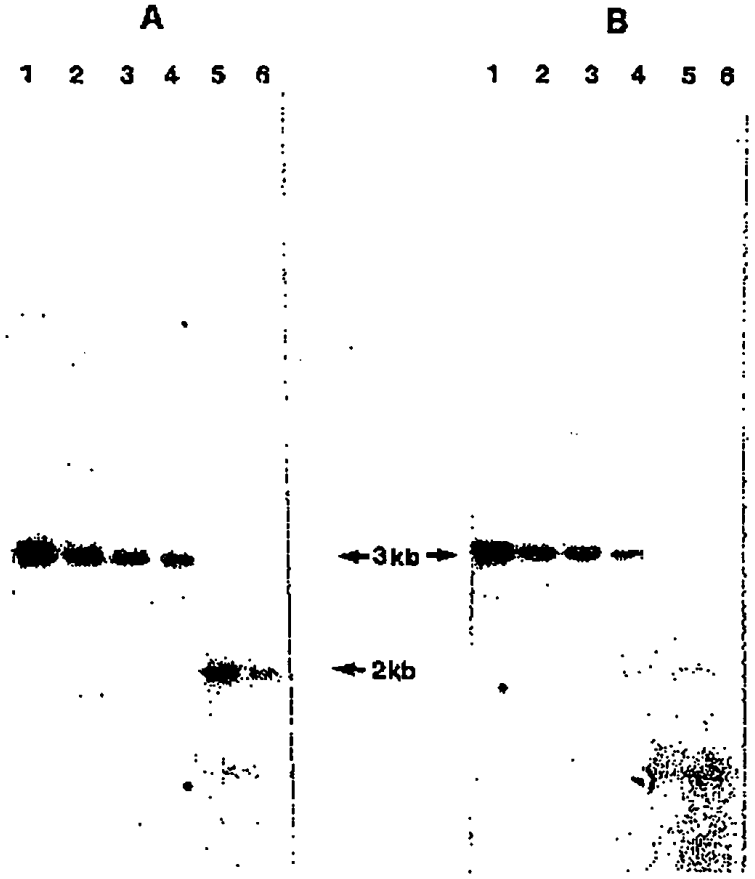


FIGURE 6

2108113

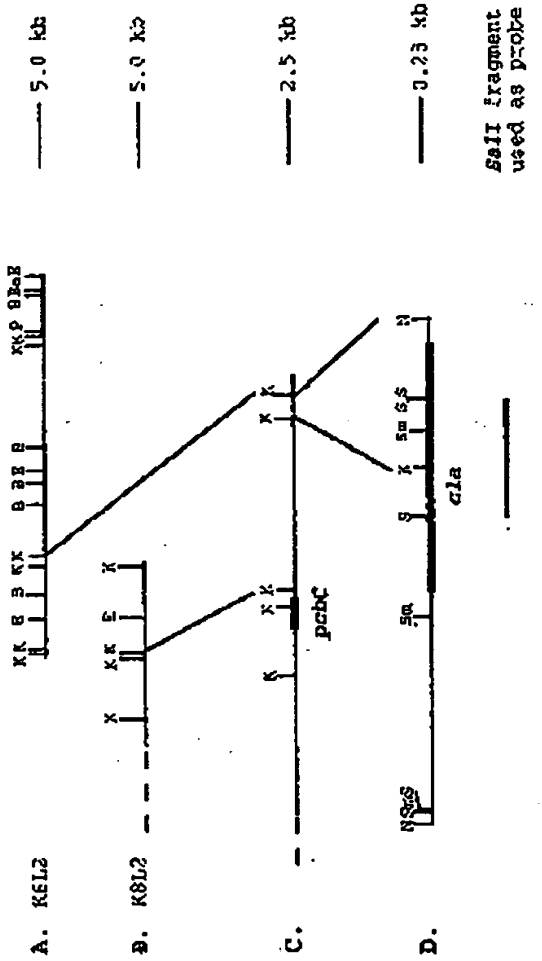
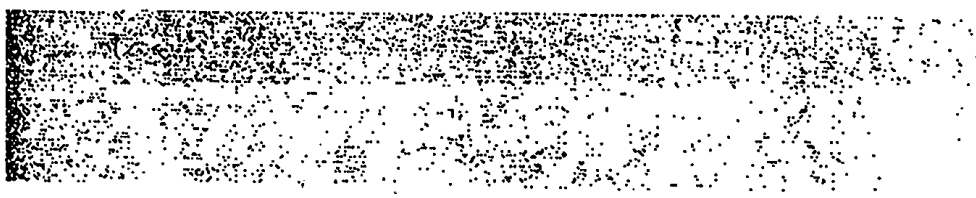


FIGURE 5

2108113

```
      |  10      |  20      |  30      |  40      |  50      |  60
   1 MIHSINYGDD PPQSRRRSRG RAATAVVAGL AVIVGLGYWG YTSLVADEKD SGDPEVEAAA 60
  61 GQFDTFLGAW EKGDAPTAAG LTDTPQNAES LIKSVMINLK PIMTRTTAKT GEKNPEGEVE 120
 121 IPFTVRNILP GAGEYAWDST AKVVGGKEW KVAFNTEMIH PQMVPGQTLA LKSRERADIL 180
 181 DANGNVLQAA SIIGAVDPRT GKGEAGLQGR YDKQLTGGSG AARSVVILDR EGGQVVKKLT 240
 241 GLKDTEGKPV KTTIDPRVQS AAAAALEGSK KNAAIVAVDP ATGNILAAAN VPSGMNRALE 300
 301 GRYPPGSTPK VVTTAALAQQ GMNPEERADC PKFAHVNGQS PENQDQFTLP AGSTFRDSPA 360
 361 HSCNTFFVNS RSKLSESSLK QAAEAFGIGG TWDVGRSTFD GSVPVSNSEN GKAASTIGQA 420
 421 RVEASPLAMA SIAATVKQGE FKQPVLVPDA VKKPHQAPRM APGIVDSLRS MMRSTVTDGA 480
 481 GDALRGLGGQ PHAKTGTAEF GTEKPPKTHA WMIGYQGDRM IAMSVLLEDG GSGGADAGPV 540
 541 AAKFLSNLAA G2                                                   552
      |  10      |  20      |  30      |  40      |  50      |  60
```

FIGURE 10



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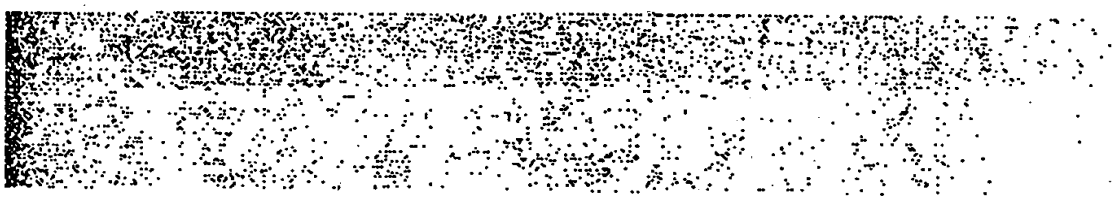
```
      |  10      |  20      |  30      |  40      |  50      |  60
  1 MTHSDNYGDD PFQGRRRSRG RAATAVVAGL  AVTVGLGYWG YTSLVADEKD SGDPEVEAAA 60
 61 GQFDTFLGAW EKGDAPTAAG LTDTPDNAES  LIKSVMINLK PTNTRTTAKT GESKNPEGEV 120
121 IPFTVRMTLP GAGEYAWDST AKVVGGGKEW  KVAFNTEMIH PQKVPGQTLA LKSREEADIL 180
181 DANGNVLQAA SIIGAVDPRT GKGEAGLQGR  YDKQLTGGSG AARSVVILDR PSGQVVKKLT 240
241 GLKDTEGKPV KTTTDPRVQS AAAAALEGSK  KNAAIVAVDP ATGNILAAAN VPSGMNRALE 300
301 GRYPPGSTFK VVTTAALQQQ GMNPEERADC  PKFAKVNGQS PENIDQFTLP AGSTFRDSFA 360
361 HSCNTFFVNS RSKLSESSLK QAAEAFGIGG  TWDMGASTFD GSVPVSNSEN DKAASTIGQA 420
421 RVEASPLVMA SIAATVRQGE FKQPVLVPDA  VKKPHQAPRM APGIVDSLRS MMRSTVTDGA 480
481 GDALRGLGGQ PHAKTGTAEF GTEKPPKTHA  WMIGYQGDRN IAMSVLLEDG GSGGADAGPV 540
541 AAKFLSNLAA GZ                                                    552
      |  10      |  20      |  30      |  40      |  50      |  60
```

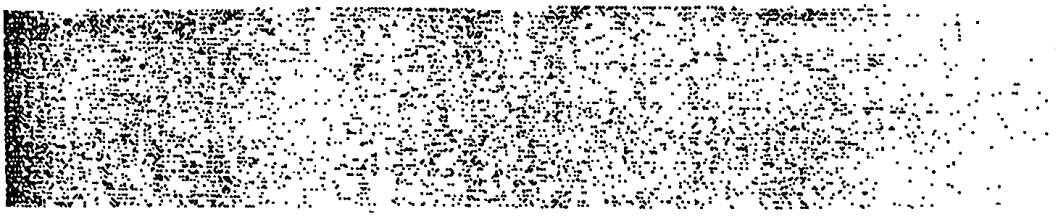
FIGURE 10



2108113

```
      |  10      |  20      |  30      |  40      |  50      |  60
   1 MERVSTAPSG KPTAAHALLS RLRDHGVGKV PGVVGREAAS ILPDEVDPID FVLTRHSFTA 60
  61 GVAADVLARI TGRPQACKAT LGPQMTNLST GIATSVLDRS PVIALAAQSE SRDIFPNDTH 120
 121 QCLDGVAIVA PMSLYAVSLQ RPHEITDLVD SAVNAAMTSP VGPSFISLPV DLLGSEEGID 180
 181 TTVFNPPANT PAKPVGVVAD GWQKAADQAA ALLAEAKHPV LVVGAAAIRS GAVPAIRALA 240
 241 ERLNLPVITT YIAKGVLPVG HELNYGAVTG YMDGILNFPA LQTMPAPVDL VLTVGYDYAE 300
 301 DLRPSMWQKG IEKKTVRISP TVNPIPRVYR PDVDVVTDVL APVEHFETAT ASPGAKQRHD 360
 361 IEPLRARIAE FLADPETYED GMRVHQVIDS NQTVMEEAAE FGDSTIVSDI GPSRMYGVLF 420
 421 ARADQPFGFL TSAGCSSFGY GIPAAIGAQM ARPDQPTFLI AGDGGFHSNS SDLETIARLN 480
 481 LPIVTVVVNN DINGLIELYQ NIGHHRSHDP AVKFGGVDFV ALAHAMGVDA TRATNRSELL 540
 541 AALRKGAELG RPFLIEVPVN YDFQPGGFGA LSIZ                          574
      |  10      |  20      |  30      |  40      |  50      |  60
```

FIGURE 11



2108113

```
      (  10      (  20      |  30      |  40      |  50      |  60
  1 MGAPVLPAAF GFLASARTGG GRAPGPVFAT RGSHTDLDTP QGERSLAATL VHAPSVAPDR 60
 61 AVARSLTGAP TTAVLAGEIY NRDELLSVLP AGPAPEGDAE LVLRLLERYD LHAFRLVNGR 120
121 PATVVRTGDR VLLATDHAGS VPLYYCVAPG EVRASTEAKA LAAHRDPKGF PLADARRVAG 180
181 LTGVYQVPAG AVMDIDLGSG TAVTHRTWTP GLSRRILPEG EAVAAVRAAL EKAVAQRVTP 240
241 GDTPLVVLSG GIDSSGVAAC AHRAAGELDT VSMGTDTSNE FREARAVVDH LRTRHREITI 300
301 PTTELLAQLP YAVWASESVD PDIIEYLLPL TALYRALDGP ERRILTGYGA DIPLGGMHRE 360
361 DRLPALDTVL AHDWATFDGL NENSFVLSTL AGHWTTHPYW DRSVLDLLVS LEAGLKRRHG 420
421 REKWVLRAAM ADALPAETVN RPKLGVHEGS GTTSSFSRLL LDNGVAEDRV HEAKRQVVRE 480
481 LFDLTVGGGR HRSEVDTDDV VRSVADRTAR GAAZ                          514
      |  10      |  20      |  30      |  40      |  50      |  60
```

Figure 12

2108113

```
      |  10      |  20      |  30      |  40      |  50      |  60
  1 VERIDSKVSP RYAQIPTFVR LPHDPQPRGY  DVVVIGAPYD GGTEYRPGVR PGPQAIRSES 60
 61 GLIKGVGIDR GPGTFDLINC VDAGDINLTP  FDMNIAIDTA QSHLSGLLKA NAAFLMIGGD 120
121 HSLTVAALRA VAEQNGFLAV VHLDAHSDTN  PAFYGGRYHH GTPFRHGIDE KLIDPAAMVQ 180
181 IGIRGHNPKP DSLDYARGHG VRVVTADEFG  ELGVCGTADL IREKVGQRPV YVSVDIDVJD 240
241 PAFAPGTGTP APGGLLSREV LALLRCVGDL  KPVGFDVMEV SPLYDHGGIT SILATEIGAE 300
301 LLYQYARAHR TQLZ                                                   314
      |  10      |  20      |  30      |  40      |  50      |  60
```

FIGURE 13

2108113

```
      |  10      |  20      |  30      |  40      |  50      |  60
  2 MASPIVDCTP YRDELLALAS ELPEVPRALL HGPLDEAKTL AARLPEGLAA ALDTFNAVDS 60
 61 EDGYLLLRGL PVDDSELPET PTSTPAPLDR KRLVMEAMLA LAGRRLGLWT GYQELRSGTV 120
121 YHDVYPSPGA HYLSSETSST LLEPHTEMAY HILQPNYVML ACSRADMER AETLVCSVRK 180
181 ALPLLDEKTR ARLFDRKVPC CVDVAFRGGV DDPGALAAVK PLYGDANDPF LGYDRELLAP 240
241 EDPADKEAVA KLSQALDDVT VGVKLVPGDV LIIDNFRTTH ARTPFSPRWD GRDRMLHRVY 300
301 IRTDSNGQLS GGERAGDTIS FSPRRZ                                    326
      |  10      |  20      |  30      |  40      |  50      |  60
```

FIGURE 1A

2108113

```
      |  10      |  20      |  30      |  40      |  50      |  60
  1 MSDSTPRTPR GPVVHTAPVG LADDGRHDPT VLASTAPATV SAVFTRSRPA GPSVVLCRSA 60
 61 VADGQARGVV VLARNANVAT GLEGEENARE VREAVARALG LPEGEMLIAS TGVIGRQYPM 120
121 ESIRERLKTL EWPAGEGGFD RAARAIMTTD TRPKEVRVSV GGATLVGIAK GVGMLEPDMA 180
181 TLLTPFATDA RLDPAEQDRL FRRVMDRTFN AVSIDTDTST SDTAVLFANG LAGEVDAGEF 240
241 EEALRTAALA LVKDLASDGE GAAKLIEVQV TGARTDAQAK RVGKTVVNSP LVKTAVHGCD 300
301 PNWGRVAMAI GKCSDDTDID QERVTIRFGE VEVYPPKARG DQADCALRAA VAEHLRGDEV 360
361 VIGIDLAIAD GAFTVYGCDL TDGYVRLNSE YTTZ                          394
      |  10      |  20      |  30      |  40      |  50      |  60
```

FIGURE 15

2108113

```
      |  10      |  20      |  30      |  40      |  50      |  60
  1 METTRSTTAD EGFDAGVRGV VAPTDAPGGT LRLVRTDQFD SLDPGNTYYA YTWNFLRLIG 60
 61 RTLVTFDTAP GKAGQRLVPD LAESLGEBSE DGRVNTYRLR EGLRYEDGTP VVSADIKHAI 120
121 ARSNYGTDVL GAGPTYFRHL LGTEYGGPWR EPDADGPVTL ETPDERTLVF RLREPFAGMD 180
181 LLAIMPETTP VPRDRDTGAE YRLRPVATGP YRIVSYTRGE LAVLEPNPHW DPETDPVRVQ 240
241 RASRIEVHLG KDPHEVDRML LAGEAHVDLA GFGVQPARQE RILAEPELRA HADNPLTGFT 300
301 WIYCLSERIA PFDNVHCRRA VQFATDKAAM QEAYGGAVGG DIATTLLPPT LDGYKHFDRY 360
361 PVGPEGTGDL EAARAELKLA GMPDGFRTRI AARKDRLKEY RAAEALAAGL ARVGIEAEVL 420
421 DFPSGDYFDR YGGCPEYLRE HGIGIDMFGW GADFGDGYGF LQQITDGRAI KERGNQNMGE 480
481 LDDPEIMALL DEGAQCADPA RRAEIWHRID QLIMDHAVIV PYLYPRELLY RHPDTRNAFV 540
541 TGSFGMYDYV ALGAKZ                                              556
      |  10      |  20      |  30      |  40      |  50      |  60
```

FIGURE 16

2108113

```
      |  10      |  20      |  30      |  40      |  50      |  60
   1 MEVARRTGVR HGIVERRLDR LDRIVQLPLT LRSEHTARLT TAGSRILVAG RRFFHQVDLA 60
  61 ARTHIFGHGS EAVDAPEVLS LVSTEPLLDS VVEDRAASLD LLLSVREEAP HQVAAQLAGY 120
 121 QVDAAYTWSL QSPRHSLSRS VRTCEVLDDP LWVILPRDHP LAARREVSLA DLRDRTWVSE 180
 181 TGPGSEILVT RVFQLAGLTA PTRIHITGAS VARGILRAGD AIGIGSPTHP AVQDFSLVRR 240
 241 SLAERPERTT SLNVDPTIVP RALAGRLAAL IAEVQLRRFA EHHRDLLDEP WWAQWYAEET 300
 301 GADARRFGAG PDQQSVPGQA EGRKLDVDDL HLLQAVARHG SINRAAAVLS LSQSALTRRI 360
 361 KRLEQSLGAR LLLRSPRGTS LTGPTRQFLR QLALYEAEFR EAALACRSVE RFLAQGWNPI 420
 421 REGVRAGARN SGZ                                                  431
      |  10      |  20      |  30      |  40      |  50      |  60
```

FIGURE 17

2108113

```
      |  10      |  20      |  30      |  40      |  50      |  60
  1 MPSALQGKVA LITGABBJIG BATARALAAE  GAAVAIAARR VEKLRALGDE LTAAGAKVHV 60
 61 LELDVADRQS VDAAVASTVE ALGGLDILVN  NAGIMLLGPV EDADTTDWTR MIDINLLGLM 120
121 VMTRAALPHL LRSKGTVVQN SSIAGRVNVR  NAAVYQATKF GVNAFSETLR QEVTERGVRV 180
181 VVIEPGTTDT ELRGHITHTA TKEMYEQRIS  QIRKLQAQDI AEAVRYAVTA PHHAIVKEIF 240
241 IRPTDQVZ                                                         248
      |  10      |  20      |  30      |  40      |  50      |  60
```

FIGURE 18

2108113

```
      |  10      |  20      |  30      |  40      |  50      |  60
  1 MMNRAAPQSD QVAPAYEMKR VCPVDPPQL  AGLRSQKAAS RVTLWDGSQV WLVTSHAGAR 60
 61 AVLGDRRFTA VTSAPGPPML TRTSQLVRAN PESASFIRMD DPQHSRLRSK LTRDPLARRA 120
121 EALRPAVRSL LDEILGGLVK GERPVDLVAG LTIPVPSRVI TLLFGAGDDR REFIEDRSAV 180
181 LIDRGYTPEQ VAKARDELDG YLRELVEERI ENPGTDLISR LVIDQVRPGK LRVEEMVPMC 240
241 RLLLVAGHGT TTSQASLSLL SLLTDPELAG RLTEDPALLP KAVEELLRTH SIVQNGLARA 300
301 AVEDVQLDDV LIRAGEGVVL SLSAGNRDET VFPDPDRVDV DRDARRHLAF GHGSHQCLGQ 360
361 WLARVELEEI LAAVLRWMPG ARLAVPFEEL DFRHEVSSYG LGALPVTWZ           409
      |  10      |  20      |  30      |  40      |  50      |  60
```

FIGURE 19